EXPERIMENTAL STUDIES ON TUMOUR-INDUCING VIRUSES OF FOWLS

by

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RESEARCHES UPON THE VIRUS-INDUCED TUMOURS OF FOWLS

Introduction

The investigations reported in the body of the thesis were initiated at the Lister Institute, London, in November 1937 in collaboration with Dr C.R. Amies, a special grant having been obtained from the British Empire Cancer Campaign for the specific study of fowl tumour viruses.

The programme of work at the Lister was directed to the evolution of biochemical and biophysical techniques for the study of these viruses since such methods had proved their usefulness with plant viruses and vaccinia.

Though the main requirement for such techniques, the rapid and easy production of large quantities of virus, was successfully met, the association of large amounts of inert material of similar chemical and physical properties to the virus, previously unsuspected, limited the investigation to immunological methods (1).

An interesting finding derived from this early study was the demonstration that the Des Ligneris sarcoma and the Fujinami sarcoma were indistinguishable; this was the more remarkable in view of the origin of the former from chemically treated tissue cultures, while the latter is a naturally occurring neoplasm (2).

The fowls used for these researches were derived from the Brown Leghorn flock of Dr Greenwood, maintained at the Institute of Animal

Genetics, Edinburgh. The varying susceptibilities of the individuals noted during the experiments were found from breeding data supplied by Dr Greenwood to have a genetic basis. A special grant was obtained to study this point, and this work was started in 1939. At this time Dr Amies was appointed to direct the Serum Department at Elstree, and ceased his connexion with the research programme.

The investigations were resumed at the Institute of Animal Genetics, Edinburgh. The different type of facilities here available resulted in a redirection of the work away from the chemical and physical aspects to a more biological viewpoint. The discovery of the recurring tumours led to the recognition of previously unsuspected carriers of tumour virus in fowls, and attempts were made to evaluate their importance as sources of infection in poultry flocks (3,8,11, 17).

An explanation for the absence of the virus in extracts of such recurring tumours was also required. It was thought probable that the puzzling variations in the yield of virus from different tumours might be due to related causes, so parallel investigations were undertaken on these two lines. This suspicion proved correct; the absence of the virus in extracts of recurring tumours was found to be an extreme case of the variation normally found in tumours, and this variation was found to be due to a reduction in the activity of the virus due to antibodies to the virus present in the extra-cellular portions of the tumour which neutralised the virus when the tumour

cells were disrupted in the preparation of tumour extracts (7).

In this manner was explained not only the variations in the infectivity of extracts, which thus became predictable as they depended upon the duration of the stimulus to antibody formation, but also the so-called "inhibitor" action of certain tumour extracts sometimes noted, but never adequately accounted for, by other workers. It was proved that the "inhibitor" action, in all cases that were investigated, was due to an excess of antibody over that needed to neutralise the virus, and that it was a response of the host, and not of the tumour (9).

This extracellular antibody was also found to be the cause of the variation in the amount of virus disseminated from a tumour to the organs of the host, or to a non-filterable tumour growing in the same host. It had previously been suggested that the cells of these organs or the non-filterable tumour were infected by the disseminated virus, but this was shown not to be the case (10, 14).

This work on the antibody was also important in the general theory of cancer, as it indicated that only early tumours could be expected to yield virus, and thus experiments designed to prove the absence of virus in any tumour were invalid if old growths were used.

In connexion with the breeding experiments on susceptibility to tumour virus, the reactions of over 1000 fowls were tested by inoculation with the Rous No.1 virus. A resistant line was produced, exceedingly interesting as its resistance is directed against developing tumours, a point of considerable interest from the point

of view of possible therapeutic applications. Such inherited resistance is also important to the poultry industry, in which losses due to neoplastic diseases are heavy. A preliminary study of the nature of this resistance has been published, and also data upon its independence of seasonal fluctuations (5, 6).

A number of the tumour-bearing birds obtained during this work were used for chemotherapy tests. The most important result was the recognition of the synergistic action between the viruses and chemical carcinogens (4, 12).

Some time had been devoted to a study of the nucleic acid variations in the fowl tumours in relation to virus activity and host susceptibility. The final result was to throw doubts on the specificity of the Feulgen reaction used for the determination of desoxyribosenucleic acid in cells (13).

The series ends with a paper discussing the discrepancy between the actual and expected yields of virus from fowl tumours, the nature of the associated inert material, and the importance of these points in connexion with the theory of cancer (15).

INDEX TO PAPERS.

1. Immunological experiments with highly concentrated suspensions of the Rous 1 tumour-producing agent. J.Path.Bact., 49, 497, 1939. (with Dr C.R. Amies).
2. Experiments on the Des Ligneris fowl sarcoma. Amer.J.Cancer, 35, 72, 1939. (with Dr C.R.Amies and Dr W.J. Purdy).
3. Observations upon spontaneously recurring Rous No.1 tumours. Brit.J.Exp.Path., 23, 206, 1942.
4. The effect of some substances influencing cell activity upon the growth of the Rous No.1 sarcoma. Brit.J.Exp.Path., 23, 221, 1942.
5. The absence of a seasonal influence upon the Rous No.1 sarcoma in young chicks. Brit.J.Exp.Path., 23, 339, 1942.
6. Some investigations into the nature of the resistance of an inbred line of fowls to the development of the Rous No.1 sarcoma. Brit.J.Exp.Path., 24, 127, 1943.
7. The relation between age, structure, and agent content of Rous No.1 sarcomas. Brit.J.Exp.Path., 24, 133, 1943.
8. Prolonged antibody production following recovery of fowls from Rous No.1 sarcoma. Brit.J.Exp.Path., 24, 138, 1943.
9. Experiments on the inhibitor occurring in Rous No.1 sarcomas. Brit.J.Exp.Path., 25, 46, 1944.
10. The tumour virus disseminated from Rous No.1 tumours, Proc.Roy. Soc. Edin., B.62, 51.
11. Lack of transmission of avian tumour virus from carrier hens to their offspring via the egg.Proc.Roy.Soc.Edin.,B.62,54, 1944.

12. Action of notatin on the Rous No.1 sarcoma virus. Nature,155, 202, 1945.

13. Mechanism of the Feulgen reaction. Nature, 156, 143, 1945.

14. The tumour virus disseminated from filterable to non-filterable tumours. Brit.J.Exp.Path. (in press; typescript copy submitted).

15. An unexplained discrepancy between the actual and expected yield of virus from avian tumours and its implications. Proc.Roy.Soc.Edin.B. (in press; typescript copy submitted).

16. Comparisons between the milk factor and fowl sarcoma viruses. Biochem. J., 39, ix, 1945. (Reprint not available. Typescript copy submitted).

17. Heritable susceptibility of poultry to cancer virus. Ann. Applied Biol., 32, 279, 1945.

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Immunological Experiments with Highly Concentrated Suspensions of the Rous I Tumour-Producing Agent

BY

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From the Lister Institute, London

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IMMUNOLOGICAL EXPERIMENTS WITH HIGHLY CONCENTRATED SUSPENSIONS OF THE ROUS I TUMOUR-PRODUCING AGENT.

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The problem of isolating the tumour-producing agent of the Rous I chicken sarcoma from the malignant growth to which it has given rise is one of particular technical difficulty. All methods of processing start at a disadvantage from the fact that the amount of agent relative to that of the tumour is always small and sometimes may not be demonstrable at all. Moreover many of the procedures which have been successfully employed in the purification and concentration of the viruses are inadmissible on account of the rapid inactivation of the agent by oxygen and its lability even at low temperatures. Isolation by physico-chemical methods has been attempted with some measure of success by several investigators, including Murphy and Claude and their collaborators, whose extensive investigations in this field are well known (for references see Claude and Murphy, 1933). During recent years, however, the most important advance has been the discovery by Ledingham and Gye (1935) of the particulate nature of the Rous I and Fujinami agents. Their experiments not only demonstrated that the size of the " infective " particles is comparable with that of the larger viruses such as vaccinia, but also showed that the agents can be concentrated and purified by fractional centrifugation. McIntosh (1935) shortly afterwards reported similar experiments and further confirmation is to be found in more recent communications by Amies (1937) and Claude (1937, 1938). In spite, however, of the considerable progress thus made, our present knowledge of the nature of the avian tumour agents is meagre as compared with that which we now possess of some of the plant and animal viruses. These collateral researches suggest the lines on which further investigations may profitably be pursued. The material necessary for such studies is an adequate supply of highly purified tumour agent and attention has accordingly been focussed upon the problem of providing this.

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Methods.

The majority of the experiments were carried out with a strain of Rous I sarcoma originally obtained from Dr W. E. Gye in 1935. Less frequently another filterable tumour of fowls, here designated the des Ligneris sarcoma, was employed. The relationship of this tumour, which closely resembles the Fujinami sarcoma, to other avian sarcomata has been discussed elsewhere (Amies, Carr and Purdy, 1939). The chickens used were Brown Leghorns bred specially for this work by Dr A. W. Greenwood of the Department of Animal Genetics, University of Edinburgh. These were 6-8 weeks old at the time of inoculation. All experiments were carried out as far as possible on a quantitative basis, the tumour-producing activity of each preparation being estimated by titration on groups of 3 or 4 chickens. When a comparison of the activities of the two different preparations was required, tenfold dilutions of each were inoculated intramuscularly into corresponding sites (breast muscles and legs) on opposite sides of the same bird, the test being carried out as before on groups of 3 or 4 chickens.

In order to obtain relatively large amounts of concentrated and highly purified tumour agent it is necessary to process a considerable quantity of tumour and this has made it necessary to introduce a number of modifications in the methods previously described (Amies).

Preparation of cell-free tumour extracts. Fowls bearing rapidly growing tumours are killed by injecting them intravenously with 1·0 c.c. of a molar solution of potassium cyanide. This is done in order that the cyanide may from the very outset of the process exert its effect as an inhibitor of oxidation. The tumour is removed aseptically and immediately placed in an ice-cold dish. It is then finely minced by means of a Latapie mincing machine which has been sterilised and cooled to 4° C. The usual grinding of the tumour with sand is omitted in order to prevent oxidation and to avoid the presence of finely divided particles of silica in the finished preparation. The minced tissue is next brought to a temperature of −16° C. and then rapidly thawed, this process being repeated six times in order to disintegrate the cells and liberate the tumour agent. An M/250 citric acid-phosphate buffer solution of *p*H 7·2 containing 1 : 10,000 hydrogen cyanide, cooled to 4° C. before use, is then added to the minced tumour to give a 10 per cent. suspension. This is filtered through sterile cheese cloth to remove coarse debris.

Tumour extracts prepared in this manner contain a considerable amount of finely divided cell debris which must next be removed. This is in part accomplished by means of a laboratory type Sharples centrifuge. The instrument is driven by means of an electric motor and is capable of a rotational speed of 25,000 *r.p.m.*, equivalent to a centrifugal field of about 15,000 times gravity at the periphery of the rotor or "bowl." All metal parts coming into contact with the fluid which is being centrifuged are of Monel metal. The rotating bowl, which is of the clarifier or continuous flow type, is lined with a cylinder of cellophane made from a sheet of this material $\frac{5}{1000}''$ thick. This greatly facilitates the removal of the deposit at the conclusion of the run. The bowl is cooled to 4° C. before use. The tumour extract is allowed to flow through the bowl at the rate of about 50 c.c. a minute, whereby the greater part of the cell debris is deposited while the tumour agent remains in the effluent. In this manner it is possible to prepare several litres of extract in a short space of time. Preliminary experiments showed that such extracts are highly active, the inoculation of 0·25 c.c. of a 1 : 1000 dilution usually producing a tumour within 2 or 3 weeks.

Deposition of the tumour agent by centrifugation. For the purposes for

which it is ordinarily employed, the Sharples centrifuge gives a satisfactory performance, but for research on viruses it has certain disadvantages which can only partly be overcome. If the closed or "batch" type of bowl is used the temperature of the fluid rises appreciably during centrifugation. Pollard (1938), who used a batch bowl to obtain sedimentation of the Rous I agent, found that the temperature was raised from 5° to 25° C. during a run of 45 minutes at an estimated rotational speed of 36,000 *r.p.m.* Although this upper limit of temperature is not rapidly detrimental it should be avoided if possible, and the associated convection currents set up in the fluid must to some extent offset the centrifugal force applied, particularly with the machine used in the present investigation, which has a maximal speed of 25,000 *r.p.m.* It was for these reasons that a clarifier bowl was used instead of a batch bowl. In the former there is a continuous circulation of air and no appreciable rise occurs in the temperature of the extract throughout the period of centrifugation. Contamination of the deposit by dust derived from the air circulating through the bowl is avoided by surrounding the entire machine with a cover consisting of a wooden framework panelled with sheets of celluloid or cellophane. The air passes into this cover through a cotton wool filter and is led out of it by means of wide bore rubber tubing fixed to the collector spout. The effluent also runs through a similar rubber tube and is collected in a suitable vessel. A further disadvantage of this type of centrifuge is the aeration which the fluid undergoes in its passage through the bowl. The only complete solution of this problem would be to replace the air by an inert gas but this is not at present practicable.

The next step in the process is to deposit the agent from the extract prepared in the manner described above. At first it was hoped to do this by passing the extract a second time through the Sharples centrifuge but at a much slower rate of flow. Preliminary experiments, however, showed that the deposit so obtained contained less than half of the amount of agent present in the extract. These somewhat disappointing results led to the adoption of a method originally used by Gye (1925), in which the extract is rendered acid prior to centrifugation. The hydrogen ion concentration employed is one which is sufficiently removed from the iso-electric point of the tumour agent particles to avoid complete flocculation or inactivation. The optimal *p*H was determined in the following manner. Equal volumes of citric acid-phosphate buffer (McIlvaine, 1921) of the required *p*H and 10 per cent. tumour extract were mixed and allowed to stand for one hour at room temperature. A mixture of the same extract with buffer of *p*H 7·2 was used as a control. A series of dilutions of each mixture was then prepared and inoculated intramuscularly into chickens. Separate tests were carried out at *p*H levels of 4·5, 5 and 6, and the series was later completed by the addition of several experiments in which the *p*H of the extract was adjusted by means of borate buffers to 8, 9, 10, 11 and 12. The results showed that the agent is not affected by being maintained for one hour at any hydrogen ion concentration between 5·0 and 12·0. At *p*H 4·5, however, the activity of the agent is diminished, while the addition of an equal volume of M/50 NaOH completely destroys the tumour-producing properties of an extract. The protocol of one representative experiment is given in table I. The practice is to shorten this period to 20-30 minutes because some of the protein constituents of the extract become denatured when the *p*H is maintained for some hours at this level, and ultimately a heavy flocculum is produced. Extracts prepared in the same manner from normal tissues behave similarly. A *p*H of 5·0 appears to be the optimum for the purpose required.

The tumour agent is deposited from the acid extract by passing it through the Sharples centrifuge, the rate of flow being in this case about 40 c.c. per minute. The sediment thus obtained is immediately resuspended in chilled buffer broth in the proportion of 1 c.c. for each 100 c.c. of the original tumour

TABLE I.

The stability of a Rous I cell-free tumour extract at pH 5·0.

Site.	Inoculum.	Fowl 653.	Fowl 654.	Fowl 655.
R. breast .	1 vol. of 10 per cent. tumour extract + 1 vol. of buffer of *p*H 7·2	++++	+++	++++
L. ,, .	1 vol. of 10 per cent. tumour extract + 1 vol. of buffer of *p*H 5·0	+++	+++	++++
R. leg .	Mixture of extract + *p*H 7·2 buffer, diluted 1 : 10	+++	+++	+++
L. ,, .	Mixture of extract + *p*H 5·0 buffer, diluted 1 : 10	++	+	+++
		Fowl 656.	Fowl 657.*	Fowl 658.*
R. breast .	Mixture of extract + *p*H 7·2 buffer, diluted 1 : 100	+++	++	+
L. ,, .	Mixture of extract + *p*H 5·0 buffer, diluted 1 : 100	+++	++	+
R. leg .	Mixture of extract + *p*H 7·2 buffer, diluted 1 : 1000	++	−	−
L. ,, .	Mixture of extract + *p*H 5·0 buffer, diluted 1 : 1000	++	−	+

* The tumours in these fowls subsequently regressed and had completely disappeared when the birds were killed and examined. In the others the number of plus signs indicates the size of the tumour at death. The extract was mixed with the buffer solutions and kept at room temperature for one hour before inoculation. The volume of the inoculum was 0·25 c.c.

extract. This buffer is composed of equal volumes of plain nutrient broth and McIlvaine's buffer of *p*H 7·2. The resulting heavy suspension is then clarified in a horizontal centrifuge for 20 minutes at 2000 *r.p.m.* The deposit is again suspended and clarified and the two supernatants are pooled.

Further purification by means of proteolytic enzymes. Tumour agent suspensions prepared by these methods were satisfactory as regards their activity but they still failed to reach the degree of purity necessary for accurate serological and physico-chemical analysis. Repeated fractional centrifugation does not succeed in separating completely the agent from finely particulate material derived from the disintegration of the tissue cells. Consideration was therefore directed to the possibility of removing such debris by digesting it with trypsin or other proteolytic enzyme. This method has already proved of service in the purification of myxoma virus (Rivers and Ward, 1937), vaccinia (Smadel and Wall, 1937) and bacteriophage (Northrop, 1937-38).

The ability of the Rous I agent to withstand the action of pancreatic extracts was already known from the work of Baker and McIntosh (1927). It was only necessary, therefore, to ascertain the conditions under which effective proteolysis of the extraneous material might be secured without detriment to the agent itself. For this purpose a number of experiments were carried out with tumour agent suspensions partially purified by fractional

centrifugation. The effect on these of various concentrations of trypsin was determined, the preparations so treated being subsequently titrated on chickens in the usual manner. Digestion at different hydrogen ion concentrations was also studied. The suspending medium was nutrient broth with an equal volume of citric acid-phosphate buffer of the required *p*H. Merck's " pancreatin absolute " was used as the source of the ferment and digestion was usually carried out at 37° C. These preliminary experiments indicated that a concentration of 0·1 per cent. of pancreatin at *p*H 9·0 could be used for periods up to 3 hours without marked deterioration of the tumour-producing activity of the suspensions. The results of one such experiment are given in table II. Varying the concentration of

TABLE II.

The effect of commercial trypsin on a Rous I tumour agent suspension.

Site.	Inoculum.	Fowl 734.	Fowl 735.	Fowl 736.
R. breast .	Rous I T.A.S. + trypsin	++++	++++	++++
L. ,, .	Rous I T.A.S. without trypsin	++++	++++	++++
R. leg .	Rous I T.A.S. diluted 1 : 10 + trypsin	++	+++	++
L. ,, .	Rous I T.A.S. diluted 1 : 10 without trypsin	++	+++	+++
		Fowl 737.	Fowl 738.	Fowl 739.
R. breast .	Rous I T.A.S. diluted 1 : 100 + trypsin	++++	+++	+
L. ,, .	Rous I T.A.S. diluted 1 : 100 without trypsin	++++	++++	++
R. leg .	Rous I T.A.S. diluted 1 : 1000 + trypsin	+++	+	−
L. ,, .	Rous I T.A.S. diluted 1 : 1000 without trypsin	−	+	+

T.A.S. = tumour agent suspension.

The number of plus signs indicates the size of the tumour at death. The T.A.S. was prepared in a mixture of equal volumes of nutrient broth and M/5 Na_2HPO_4 (*p*H 9·0). To one half of this 0·2 per cent. of Merck's pancreatin was added, the other half acting as control. The T.A.S. was incubated at 37° C. for 1½ hours, the required dilutions were made and 0·25 c.c. of each inoculated.

pancreatic extract between 0·1 and 1·0 per cent. was found to have no appreciably different effect upon the tumour agent, at least during the relatively short periods of digestion employed. Owing to the lability of the agent prolonged digestion under conditions optimal for ferment action was not possible, but even the mild treatment allowable produced a noticeable clearing of the suspension. Several attempts to obtain further purification by prolonged treatment under anaerobic conditions were unsuccessful.

The satisfactory results of these experiments led to the adoption of tryptic digestion as a routine measure. The deposit obtained by centrifugation of the acid tumour extract is suspended in phosphate buffer broth of *p*H 9·0 and sufficient pancreatin is added to give a concentration of 0·1 per cent. After incubation for one hour at 37° C. the suspension is centrifuged for one hour on an angle centrifuge and the deposit so obtained resuspended in buffer solution or broth. This represents the finished suspension. In contrast with the behaviour of vaccinia elementary bodies

the Rous I "bodies" do not flocculate in the presence of physiological saline but their infectivity rapidly disappears unless the dispersing medium contains broth or some other protective colloid.

By this procedure it has been possible to prepare with fair regularity tumour agent suspensions having an activity at least as great as that of the original extract when reconstituted to the same volume. Most workers agree that a 5 per cent. cell-free tumour extract seldom contains more than 1000 minimal tumour-producing doses of agent per c.c. (20,000 doses per gram of tumour tissue). The following results, also expressed in terms of minimal tumour-producing doses per gram of tumour, were obtained in 22 consecutive experiments in which the methods here described were used.

480,000	minimal	tumour-	producing	doses	per gram of tumour,	1 experiment.
160,000	,,	,,	,,	,,	,,	1 ,,
80,000	,,	,,	,,	,,	,,	12 experiments.
16,000	,,	,,	,,	,,	,,	1 experiment.
8,000	,,	,,	,,	,,	,,	2 experiments.
4,000	,,	,,	,,	,,	,,	1 experiment.
800	,,	,,	,,	,,	,,	1 ,,
2	,,	,,	,,	,,	,,	1 ,,
					Inactive suspension,	2 experiments.

The method makes it possible to process as much as 100 g. of tumour in one batch and, since the deposit may be suspended in a few c.c. of fluid, it follows that a considerable degree of concentration can be effected. On several occasions suspensions containing one million tumour-producing doses per c.c. have been obtained.

Examination of tumour agent suspensions with the dark-field microscope. The uses and limitations of this method have been described elsewhere (Amies). The newer and more concentrated suspensions when studied in this manner usually appeared less homogeneous than the earlier preparations, in which reliance was placed entirely upon fractional centrifugation. The most probable explanation is that the alteration of the *p*H of the extract causes denaturation and flocculation of some of the tissue proteins. Extracts of normal fowl tissues, particularly liver, when treated in the same manner give rise to suspensions which closely resemble in optical properties those prepared from fowl sarcomata. A large proportion of the material obtained by the acid centrifugation method consists of particles of low optical density which an experienced observer can readily differentiate from the particles of uniform optical characters which Ledingham and Gye, McIntosh, and ourselves have come to regard as the causal agents. In this connection it is instructive to observe, under the dark-field microscope, the effect of adding undiluted rabbit anti-fowl serum to a tumour agent suspension. Within a few minutes the extraneous material becomes agglutinated, forming amorphous clumps within which are embedded a few of the more luminous "elementary bodies," but the majority of the latter remain in suspension. This presents a contrast to the effect produced by adding a Rous-immune fowl serum to the same tumour agent suspension, for in this case the "elementary bodies" are agglutinated and the extraneous material remains dispersed.

Attempts to assess the purity of the tumour agent suspensions. Each step in the process described above contributes to the purification as well as to the concentration of the agent. It is, however, a matter of considerable difficulty to assess the purity of the final product with any degree of accuracy. Viruses such as tobacco mosaic or vaccinia appear to be entirely foreign

to and do not exist in the normal healthy plant or animal but the Rous I and Fujinami tumour agents are apparently related antigenically to some constituent of normal fowl cells. This subject has already been discussed in detail (Amies) and further experiments on it are given later in this communication. Serological methods of differentiating the tumour agent from cell debris are considerably complicated by this fact. Attention is therefore being concentrated at present on physical methods, and in particular on the use of the Svedberg centrifuge, as a means of determining to what extent the tumour agent suspensions consist of particles of the same size and density. The majority of the suspensions prepared by the new methods were obviously inhomogeneous when examined by the dark-field microscope, but a few which appeared to be satisfactory were studied in this manner. One of these was found to sediment with the formation of a poorly defined boundary, indicating that the particles in it were at least approximately uniform and the sedimentation velocity of this boundary was such as might be expected from a particle of the size of the Rous I agent. On the available evidence it must be admitted that the majority of these tumour agent suspensions have not reached the high standard of purity required for exact physical or chemical analysis. Nevertheless, the methods here advocated represent an improvement upon previous processes, particularly in regard to the amount of tumour agent which can be obtained. They were successfully employed in many of the immunological experiments now to be described.

Immunological investigations.

In the first part of this communication emphasis was laid upon the importance of using highly purified suspensions of the fowl tumour agents for immunological experiments. The main reason given for this insistence was the fact that these agents are antigenically related to some constituent of normal fowl tissues. This statement is based upon the fact, first established by Gye and Purdy (1931) and subsequently confirmed in this laboratory (Amies, 1937) that antisera produced by inoculating rabbits with normal fowl protein (chick embryo or citrated whole blood) are capable of neutralising the Rous I and Fujinami sarcoma agents *in vitro*. Gye and Purdy extended their investigations to anti-fowl sera obtained from ducks, goats and horses and were able to demonstrate similar neutralising properties in these. It is of the utmost importance to determine the significance of this phenomenon because, so far as we are aware, there is at present no parallel to it in the field of immunity to infective agents. The further investigations now to be described are for this reason concerned with various aspects of this problem.

Experiments with rabbit anti-fowl sera.

Is this inhibition of the tumour agent by anti-fowl serum a true antigen-antibody reaction or is it brought about indirectly ? One possible explanation, for example, is that the agent becomes immobilised within a precipitate resulting from a combination of anti-fowl antibodies with fowl protein derived from the tumour

cells, whereby the agent is prevented from reaching susceptible cells when the mixture is inoculated. Gye and Purdy met this objection by demonstrating that neutralisation would take place without visible flocculation, and they further showed that purified preparations of the agent, prepared by adsorption and elution, were inactivated as readily as crude tumour extracts. Furthermore, suspensions of the tumour agent obtained by fractional centrifugation are also neutralised by anti-fowl serum (Amies). This evidence should be sufficient to justify the belief that the reaction is a specific one, but in view of the importance of establishing the fact beyond dispute the following additional experiments were undertaken.

The agent can be recovered in active form from a neutral mixture of tumour agent suspension and anti-fowl serum. A tumour agent suspension, prepared in the usual manner, was mixed with an equal

TABLE III.

The dissociation of a neutralised mixture of tumour agent suspension and anti-fowl serum by high-speed centrifugation.

A. Test for tumour-producing activity of deposits.

Site.	Inoculum.	Fowl 780/36.	Fowl 781/36.	Fowl 782/36.	Fowl 783/36.
	Resuspended deposit from mixture of T.A.S.				
R. breast	+normal rabbit serum, undiluted	+++	++++	+	++
L. ,,	+rabbit anti-fowl serum, undiluted	++++	+++	++	+++
R. leg .	+normal rabbit serum, diluted 1 : 10	++	−	+	++
L. ,, .	+rabbit anti-fowl serum, diluted 1 : 10	++	++	+++	+++

B. Test for neutralising power of supernatants.

		Fowl 1/37.	Fowl 2/37.	Fowl 3/37.	Fowl 4/37.
	Test dose of T.A.S.				
R. breast	+supernatant (= absorbed) serum	++	++	++	++
L. ,,	+unabsorbed serum diluted to correspond with the above	++	++	++	++
R. leg .	+normal rabbit serum diluted to correspond with the above	++++	++++	+++	++++

T.A.S. = Tumour agent suspension. The number of plus signs indicates the size of the tumours at death. The original mixture of T.A.S.+anti-fowl serum was shown to be inactive by inoculation into a separate group of fowls.

volume of rabbit anti-fowl serum of known potency. After it had been incubated for one hour at 37° C. this mixture was centrifuged at 15,000 *r.p.m.* for 45 minutes. The supernatant fluid and the deposit were then separated and the latter was resuspended in a volume of physiological saline equal to that of

the original mixture. An exactly similar procedure was carried out with a mixture containing the same amounts of tumour agent suspension and normal rabbit serum. The activities of the two resuspended deposits were then compared by inoculating a series of dilutions of each into chickens and the neutralising power of each supernatant was compared with that of the untreated anti-fowl serum. The results (table III) indicated that the high-speed centrifuge is capable of separating a neutral serum-agent mixture into its two active components. A similar phenomenon has been described by Sabin (1935) in the case of vaccinia, pseudorabies and B virus. In the present instance the result appears to render the mechanical imprisonment theory untenable, for the union of anti-fowl antibody with fowl protein is not a reversible process.

The deliberate formation of a serum protein precipitate in a suspension of the tumour agent does not affect the activity of the latter. If the mechanical theory were correct it should be possible to demonstrate that any precipitate resulting from the union of an antigen with its corresponding antibody will, at the moment of its formation, adsorb and immobilise the tumour agent. In order to ascertain whether this was the case a Rous I tumour agent

TABLE IV.

The activity of the tumour agent is not influenced by the formation of a protein precipitate in the suspending medium.

Site.	Inoculum.	Fowl 71.	Fowl 72.	Fowl 73.	Fowl 74.
R. breast	T.A.S.+normal sheep serum+rabbit anti-sheep serum. Precipitate +++	+++	+	+	+++
L. ,,	T.A.S.+normal sheep serum+normal rabbit serum. No precipitate	+++	+	+	+++
R. leg .	T.A.S. diluted 1 : 100+normal sheep serum+rabbit anti-sheep serum. Precipitate +++	+	+	−	+
L. ,, .	T.A.S. diluted 1 : 100+normal sheep serum+normal rabbit serum. No precipitate	+	+	−	+

T.A.S. = tumour agent suspension. In each case the mixture consisted of equal volumes of tumour agent suspension, normal sheep serum (1 : 20 dilution) and rabbit serum (1 : 10 dilution). The volume inoculated was 1·0 c.c. The number of plus signs indicates the size of the tumour at death.

suspension was added to a 1 : 20 dilution of normal sheep serum. To this mixture was then added a rabbit anti-sheep serum of high precipitin titre. The two sera were used in the proportions which a preliminary test had shown to give maximal flocculation. As a control test an equal volume of the mixture of tumour agent suspension and normal sheep serum was added to a similar amount of normal rabbit serum. Separate mixtures containing two different

concentrations of tumour agent with equal amounts of the other two reagents were prepared in this manner. These mixtures were kept at room temperature for one hour and then overnight in the cold room. On the following morning the tubes were shaken in order to distribute evenly the precipitates which had formed in the mixtures containing anti-rabbit serum and each mixture was inoculated intramuscularly into a group of chickens. The protocol of this experiment is given in table IV. The results demonstrate clearly that the tumour agent was not affected by the flocculated protein.

These two experiments therefore confirm the previous findings. On the evidence now available we consider that the inhibition of the tumour agent by anti-fowl serum should be accepted as a true antigen-antibody reaction.

The effect of complement on the neutralisation of the agent by anti-fowl sera. The experiments of Gye and Purdy indicated that the presence of complement was essential for the neutralisation of the Rous I agent by rabbit and anti-fowl serum. In this laboratory, however, it was found that the inhibitory effect of such sera was not dependent upon the presence of complement; and it was suggested that this disagreement might be due to the fact that purified tumour agent suspensions were used in our own experiments whereas Gye and Purdy employed cell-free tumour extracts. Subsequent experience proved this explanation to be correct, for it was found that a rabbit serum which neutralised a tumour agent suspension without complement would only exert its inhibitory effect on the cell-free extract from which that suspension was prepared if fresh complement were added to the mixture. The question is an important one, because it was partly on account of the difference between fowl anti-Rous sera, which do not require complement in order to neutralise the agent, and anti-fowl sera, which they believed did require complement, that Gye and Purdy based their well known concept of the dual nature of the tumour-producing complex.

The experiments on the effect of complement previously described (Amies) have been amplified by a further series of 8 similar experiments, in some of which the des Ligneris agent was employed instead of the Rous I. Each of these has fully confirmed the fact that neutralisation takes place in the absence of complement provided that the tumour agent is present in the form of a purified suspension.

Neutralisation experiments in vivo. Experiments were carried out to determine whether chickens can be rendered passively immune to the tumour agent by inoculating them with rabbit anti-fowl sera or with sera of rabbits which had been repeatedly inoculated with concentrated tumour agent suspensions. The plan

adopted was to inject 2 c.c. of undiluted serum slowly into the wing vein and then immediately afterwards to inject falling ten-fold dilutions of a tumour agent suspension into the breast and leg muscles in the usual manner. This titration of the suspension was repeated in a second group of chickens which received no serum and served therefore as controls. Death from shock following rapidly upon the inoculation of anti-fowl serum occurred in one chicken out of a total of 20 treated in this manner. One experiment gave definite evidence of protection against a small dose of agent but the same result could not be obtained when more active tumour agent suspensions were used. This is perhaps not surprising when it is remembered that these sera rapidly lose their inhibitory properties on dilution, even when the reaction is carried out *in vitro*. The effect of inoculating rabbit anti-fowl or anti-agent sera intramuscularly, either simultaneously with or a short time before the injection of tumour agent, has not yet been investigated.

Experiments with rabbit anti-tumour agent sera.

In the previous communication (Amies) it was stated that it had not been possible to demonstrate any antibodies in the sera of rabbits which had been repeatedly inoculated with active tumour agent suspensions. The larger amounts of agent made available by the newer methods enabled us to reinvestigate this problem. Four fully grown rabbits were accordingly given repeated injections of highly concentrated tumour agent suspension, at first intravenously and later intraperitoneally. The activity of the preparations was in many cases determined by titration on fowls in the usual manner. Samples of blood were taken at intervals and the sera were tested for the presence of antibodies to normal fowl protein and to the tumour agents. The hæmolysin test and the precipitin reaction were employed for the determination of anti-fowl antibodies, and neutralisation and agglutinating tests were used to demonstrate the anti-agent properties of the sera. As similar results were obtained with each of the four animals it will be sufficient to describe only one in detail.

Rabbit no. 19. This animal was immunised with large amounts of des Ligneris tumour agent obtained by means of the Sharples centrifuge. The suspensions were clarified by light centrifugation but were not trypsinised. A sample of serum collected before immunisation agglutinated fowl red cells to a dilution titre of 1 : 8 but failed to produce hæmolysis. After 6 inoculations containing altogether 256,000 minimal tumour-producing doses the serum hæmolysed fowl red cells to a titre of 1 : 128 and gave a very slight precipitin reaction with fowl serum. This same sample of serum completely neutralised 1000 minimal tumour-producing doses of a Rous I suspension and a similar amount of a des Ligneris suspension (table V, p. 508).

Rabbit no. 25. This experiment is quoted in order to demonstrate the difference in the response of the rabbit to immunisation with tumour agent suspension and with normal chick embryo. The animal received

five intraperitoneal injections of lightly clarified emulsion of normal 10-14-day chick embryos. Five days after the last inoculation the serum produced lysis of fowl red cells to a titre of 1 : 2000 and was precipitated by fowl serum

TABLE V.

Neutralisation of des Ligneris and Rous I tumour agent suspensions by rabbit anti-des Ligneris serum.

Site.	Inoculum.	Fowl 414.	Fowl 415.	Fow 416.
	des Ligneris T.A.S.			
R. breast	+undiluted anti-des Ligneris serum of rabbit 19	−	−	−
L. ,,	+undiluted normal rabbit serum	+++	+++	++++
R. leg .	+anti-des Ligneris serum of rabbit 19 diluted 1 : 10	+	++	++
L. ,, .	+normal rabbit serum diluted 1 : 10	+++	+++	+++
		Fowl 435.	Fowl 436.	Fowl 437.
	Rous I T.A.S.			
R. breast	+undiluted anti-des Ligneris serum of rabbit 19	−	−	−
L. ,,	+undiluted normal rabbit serum	+	++	+++
R. leg .	+anti-des Ligneris serum of rabbit 19 diluted 1 : 10	−	++	+++
L. ,, .	+normal rabbit serum diluted 1 : 10	−	+++	++++

T.A.S. = tumour agent suspension. The single tumour observed in fowl 435 subsequently regressed: in all other cases the number of plus signs indicates the size of the tumour at death. Each mixture consisted of equal volumes of tumour agent suspension and serum and all mixtures were incubated for one hour at 37° C. prior to inoculation. The volume injected was 0·5 c.c.

in a dilution of 1 : 512. In spite of these considerably greater anti-fowl properties, however, this serum did not neutralise the Rous I agent as effectively as the anti-tumour agent sera. The results are given in table VI.

TABLE VI.

Inhibition of a Rous I tumour agent suspension by rabbit anti-chick embryo serum.

Site.	Inoculum.	Fowl 564.	Fowl 565.	Fowl 566.
	Rous I T.A.S.			
R. breast	+undiluted anti-chick embryo serum of rabbit 25	−	+	+
L. ,,	+undiluted normal rabbit serum	+++	++	+
R. leg .	+anti-chick embryo serum of rabbit 25 diluted 1 : 10	++	+++	+
L. ,, .	+normal rabbit serum diluted 1 : 10	++++	+++	++

T.A.S. = tumour agent suspension. In the case of fowl 566 the tumours subsequently regressed; in the other two the number of plus signs indicates the size of the tumour at death. Each mixture, consisting of equal volumes of serum and tumour agent suspension, was incubated at 37° C. for one hour and then left in the cold room overnight. The volume inoculated was 0·5 c.c.

We conclude, therefore, that although it is not possible to guarantee that the suspensions used for immunising the animals were free from fowl tissue debris, these results may be accepted as evidence that the inoculation of the tumour agent does elicit the formation of specific antibodies.

Absorption experiments. Gye and Purdy noted that anti-fowl sera lost their ability to neutralise the Rous I agent after they had been absorbed with normal chick embryo tissue. This, of course, is the result which would be expected and our own experience has been similar. Of greater interest, however, is the finding of these investigators that normal chick embryo does not remove the inhibitory properties of sera obtained from animals which have been immunised with extracts of the Rous I or Fujinami tumours. If immunisation with crude tumour extracts gave rise to separate antibodies for fowl protein and tumour agent, and if these two had no antigen common to both, it would be reasonable to expect that absorption of such sera with chick embryo tissue would remove the former type of antibodies and leave their anti-agent properties intact. Our own experiments however have indicated that this

TABLE VII.

Removal of the inhibitory properties of rabbit anti-tumour agent sera by absorption with normal chick embryo tissue.

Site.	Inoculum.	Fowl 547.	Fowl 548.	Fowl 549.
	des Ligneris T.A.S.			
R. breast	+rabbit 19 anti-des Ligneris serum unabsorbed	−	−	−
L. „	+the same anti-des Ligneris serum after absorption	++++	++	+++
R. leg .	+the same anti-des Ligneris serum unabsorbed	−	−	−
L. „ .	+normal rabbit serum	++++	+++	++++
		Fowl 538.	Fowl 539.	Fowl 540.
	Rous I T.A.S.			
R. breast	+rabbit 27 anti-Rous I serum unabsorbed	+	−	+
L. „	+the same anti-Rous I serum after absorption	+++	+++	+
R. leg .	+the same anti-Rous I serum unabsorbed	+	+	−
L. „ .	+normal rabbit serum	++++	++++	++

T.A.S. = tumour agent suspension. In the case of fowl 540 the tumours subsequently regressed; in the other two the number of plus signs indicates the size of the tumour at death.

Hæmolysin titres. Rabbit 19, unabsorbed, 1 : 512; after absorption, 1 : 64.
Rabbit 27, unabsorbed, 1 : 128; after absorption, 1 : 8.
A 3 per cent. suspension of normal fowl red cells was employed.

is not the case. If tumour agent suspensions are used for immunising the rabbits instead of crude extracts, absorption of the sera with chick embryo removes their inhibitory properties despite the fact that under these circumstances the amount of fowl protein in the inoculum is certainly very greatly reduced, even though it cannot be assumed to be completely absent. This is shown by the results of absorption experiments carried out with the sera of rabbits 19 and 27 (table VII).

Before the specific nature of this absorption reaction could be accepted, however, it was necessary to exclude the possibility that the anti-agent antibody was loosely adsorbed on and carried down with the embryo pulp. Such non-specific adsorption is, of course, contrary to all experience but it seemed advisable to settle the question by direct experiment.

A rabbit which was already highly immune to normal chick embryo tissue was inoculated intracutaneously with vaccinia virus in the form of a pure suspension of the elementary bodies. Two weeks later a second inoculation with vaccinia was given in order to augment the immune response to this virus. The animal was bled 5 days after this second injection and a sample of the serum was absorbed three times with chick-embryo pulp. The anti-fowl, anti-tumour and anti-viral properties of the absorbed and unabsorbed fractions of this serum were then compared. The results were as follows.

i. Before absorption.
Anti-fowl hæmolysin titre : 1 : 1024.
Neutralisation of tumour agent : 0·25 c.c. of serum neutralised 100 minimal tumour-producing doses of Rous I suspension.
Neutralisation of vaccinia : 0·0125 c.c. neutralised 10,000 *m.i.d.* of a vaccinia elementary body suspension.

ii. After absorption with chick embryo pulp.
Anti-fowl hæmolysin titre : nil.
Neutralisation of tumour agent : no inhibition.
Neutralisation of vaccinia : unchanged.

It is evident that absorption with chick embryo removed both the anti-agent properties of the serum and its anti-fowl properties but that the antibodies for vaccinia were not affected by this procedure. The suggestion that chick embryo can remove antibodies from serum in a non-specific manner is thus proved to be incorrect. Further support for this belief is given by an experiment reported by Rhoads (1931). An anti-poliomyelitis serum prepared by injecting horses with the brains of infected monkeys was absorbed by normal monkey brain tissue. This procedure removed the anti-monkey properties of the serum but left the anti-viral activity unaffected.

An absorption experiment was also carried out with a Rous-immune fowl serum. A bird in which a Rous I tumour had grown and subsequently regressed was given a large dose of a highly

active tumour agent suspension and a sample of blood was taken one week later. A sample of the serum was absorbed three times with normal chick embryo pulp and the neutralising activity of this absorbed specimen was then compared with that of the unabsorbed serum. The results (table VIII) show that the undiluted

TABLE VIII.

The neutralising activity of a Rous-immune fowl serum is not removed by absorption with chick embryo tissue.

Site.	Inoculum.	Fowl 838.	Fowl 839.	Fowl 840.
	Rous I T.A.S.			
R. breast	+Rous-immune serum of fowl 767, undiluted	−	−	+
L. „	+normal fowl serum, undiluted	++	++	++++
R. leg .	+Rous-immune serum of fowl 767, after absorption, undiluted	−	−	+
L. „ .	+normal fowl serum, undiluted	++++	+++	++++
		Fowl 841.	Fowl 842.	Fowl 843.
	Rous I T.A.S.			
R. breast	+Rous-immune serum of fowl 767, after absorption, undiluted	+	+	+
L. „	+Rous-immune serum of fowl 767, before absorption, undiluted	+	+	+
R. leg .	+Rous-immune serum of fowl 767, after absorption, diluted 1 : 10	++	++	++++
L. „ .	+Rous-immune serum of fowl 767, before absorption, diluted 1 : 10	++	++	++++

T.A.S. = tumour agent suspension : the amount injected contained 2500 minimal tumour-producing doses. All mixtures were incubated at 37° C. for one hour prior to inoculation. In the case of fowls 841 and 842 the tumours subsequently regressed ; in the others the number of plus signs indicates the size of the tumour at death.

serum both before and after absorption neutralised 2500 minimal tumour-producing doses of a Rous I suspension. It thus appears that there is a fundamental difference between the antibodies present in fowl anti-Rous serum and those which are found in the serum of rabbits immunised either with tumour agent suspension or normal fowl protein. On this point, therefore, we are in full agreement with Gye and Purdy whose findings were based on somewhat different experimental procedures.

The inability of fowl iso-antibodies to inhibit the Rous I agent. In view of the apparent antigenic relationship between the tumour agent and some constituents of normal fowl cells it was of interest to determine whether the development in a fowl (A) of iso-antibodies for another fowl (B) would render A resistant to Rous agent obtained from a tumour in B.

The experiment was carried out as follows. Two normal Brown Leghorn pullets were bled and the serum of each was tested for the presence of agglutinins for the red cells of the other. No cross agglutination could be demonstrated at 4° C., at room temperature or 37° C. One of these fowls (A) was then given 5 injections of citrated whole blood from the other fowl (B) at approximately weekly intervals. The volumes of these injections were 4·9, 2·5, 4·5 and 6·0 c.c., the first being given intramuscularly and the rest intraperitoneally. A sample of serum obtained 9 days after the last immunising dose agglutinated the red cells of fowl B to a titre of 1 : 256. Fowl B was then inoculated with Rous I agent and the tumour which developed was used for the preparation of a tumour agent suspension. The serum of A after immunisation against B was then tested for neutralising antibodies to the tumour agent suspension prepared from B, the serum of A taken before inoculation being used as the control. The results demonstrated unequivocally that the serum of A had no inhibitory effect upon the tumour agent obtained from B. Furthermore, the tumour agent prepared from the tumour of B produced a rapidly growing and fatal tumour when inoculated into A.

Summary.

1. Methods are described for the preparation of highly active concentrated suspensions of the filterable fowl tumour agents. The procedure consists essentially in sedimenting the agent from cell-free tumour extracts by centrifugation at *p*H 5-5·5 and digesting the resuspended deposit with commercial trypsin at *p*H 9·0. The agent is recovered by further fractional centrifugation.

2. By this process a considerable degree of purification is also effected but it has not yet been possible to obtain completely homogeneous suspensions of the tumour agent.

3. The inhibition of the Rous I and des Ligneris sarcoma agents by rabbit anti-fowl serum appears to depend on a specific antigen-antibody reaction.

4. The sera of rabbits which have been repeatedly inoculated with large doses of tumour agent suspension contain neutralising antibodies for the agent and also anti-fowl hæmolysins and precipitins. The latter are present only in relatively low concentration and may have been produced in response to impurities (cell debris) present in the suspensions used for inoculation.

5. Absorption of rabbit anti-fowl or rabbit anti-tumour agent serum with normal chick embryo tissue completely removes its inhibitory properties for the tumour agent. Fowl anti-agent serum, on the contrary, is not affected by absorption with chick embryo.

6. Iso-antibodies play no part in the inhibition of the tumour agent by Rous-immune fowl serum.

7. These experiments appear to support the belief that the tumour agent has at least two antigenic components, corresponding to two antibodies, one of which is present in Rous-immune fowl serum and the other in rabbit anti-fowl serum. The findings are therefore in agreement with those reported by Gye and Purdy, but we consider that both these antigenic factors are intrinsic to the agent whereas the latter workers believed that the "fowl" factor was extrinsic.

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EXPERIMENTS ON THE DES LIGNERIS FOWL SARCOMA

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The possibility of initiating malignant changes in tissue cultures of normal cells by means of dibenzanthracene or other carcinogenic compounds must have occurred to most cancer workers. It is surprising, therefore, that so few reports on the results of such investigations have been published. Fischer (1926) claimed to have produced tumours in fowls by inoculating them with cultures of chick embryo spleen which had been grown in a medium containing tar or arsenious acid. The tissues were subsequently subcultured many times in media without the addition of these substances before they were inoculated, so that the possibility that the tumours were directly caused by tar or arsenious acid could be excluded. Direct confirmation of these results is lacking, but Carrel (1925), Murphy and Landsteiner (1925), White (1927), and others have described the production of malignant tumours in fowls following the injection of arsenious acid or tar with chick embryo pulp. Failures to obtain tumours by these or similar methods have been recorded by Pentimalli (1927), Proger (1927), Begg and Cramer (1929), and Bisceglie and di Grazia (1936).

Recently, des Ligneris (1935, 1936) has reported the induction of malignant changes in tissue cultures of normal chick fibroblasts by adding dibenzanthracene to the culture medium. The method adopted was as follows.

The dibenzanthracene was used in the form of a 0.1 per cent suspension in an aqueous medium containing lecithin. One drop of this was added to vigorous hanging-drop cultures of chick embryo fibroblasts. The cultures were fed daily with fresh medium and one drop of dibenzanthracene suspension was added at intervals of eight days. After this procedure had been kept up for four weeks the addition of dibenzanthracene was stopped, but the feeding of the cultures with fresh medium was continued for a further period of seven days. Des Ligneris concluded that at the end of this period the amount of dibenzanthracene remaining in the culture must have been infinitesimally small. It is possible, however, that some may have been absorbed, either in the form of dibenzanthracene itself or in some modified form, and was thus not removed by washing. A number of these cultures were then inoculated into the breast muscles of each of 6 young Leghorn fowls. In 5 of these no growth occurred, but in the sixth a tumour was found three weeks later at the site of inoculation. This fowl was killed on the forty-second day after inoculation. The tumour, which measured $10.0 \times 6.0 \times 4.5$ cm. was indistin-

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TABLE I: *Susceptibility of Brown Leghorn Chickens to the Tumour-producing Agents of the Rous No. 1, the Fujinami, and the des Ligneris Sarcoma*

Tumour agent	Total number of chickens inoculated	Positive		Negative
		Progressive tumours	Regressions	
Rous 1	507	463(91%)	19(3%)	25(5%)
Fujinami	118	50(42%)	20(40%)	48(17%)
Des Ligneris	164	133(81%)	28(17%)	3(2%)

guishable, both macroscopically and microscopically, from a Rous No. 1 or Fujinami sarcoma. It was transplantable without difficulty into other fowls and was later found to be transmissible by cell-free tumour extracts and desiccates. From the results of certain serological experiments which will be described later, des Ligneris concluded that this tumour was an entirely separate neoplastic entity, although it resembled the Rous No. 1 and Fujinami sarcomata in its microscopic appearance.

The present communication is concerned with the relationship of this tumour to other fowl sarcomata. No attempts have been made to repeat the experiments by which the tumour was originally obtained.

BIOLOGICAL CHARACTERS OF THE TUMOUR

The tumour was received from Dr. des Ligneris in the form of a desiccate. This was suspended in physiological saline and inoculated into the breast muscles of three Brown Leghorn chickens. One of these developed a rapidly growing sarcoma, which was used to continue the strain by cell grafts and cell-free extracts. The second bird in this group developed a slowly growing tumour which eventually killed it 116 days after inoculation, while the small tumour which arose in the third bird rapidly regressed. Brown Leghorns aged eight to ten weeks were used in all the subsequent experiments.

Transmission by Cell Grafts: Twelve chickens have been inoculated intramuscularly with 0.1 ml. of the finely minced tumour. Nine of these developed rapidly growing sarcomata and died, or were killed when moribund, on an average twenty-one days after inoculation. The remaining 3 birds showed very small tumours which completely regressed.

Cell-free Transmission: A total of 164 chickens have been inoculated with active cell-free extracts or the deposits obtained by high-speed centrifugation of such extracts. Rapidly growing tumours developed in 133 of these, while 24 showed small tumours which completely regressed, and 3 proved entirely resistant. The average duration of life for those which developed progressive tumours was twenty days. These results are compared in Table I with those obtained in earlier experiments with Brown Leghorn fowls inoculated with the Rous No. 1 sarcoma and the Fujinami sarcoma.

Post-mortem examination of the rapidly growing tumours showed them to be typical examples of a highly malignant myxosarcoma. They were soft, haemorrhagic, very mucoid, and widely infiltrating growths which frequently showed a central cavity filled with a brown or green glairy fluid containing

blood clot and fragments of necrotic tissue. Often the fluid consistency of the tissue required the use of a spoon instead of a knife to remove it from the affected area. The commencement of regressive changes, when these occurred, was marked by a rapid softening, amounting frequently to liquefaction, of the growth. The onset of this regressive process usually took place on the fifteenth to the twentieth day after inoculation and was so rapid that tumours weighing probably 15 to 20 gm. were completely resolved in the space of a week. By these characteristics the des Ligneris tumour could be differentiated from a Rous No. 1 sarcoma; but from a study of several hundred examples produced during the investigation it was concluded that the new tumour could not be distinguished from the Fujinami sarcoma either by its macroscopic and microscopic structure or by its behaviour.

The Particulate Nature of the Tumour Agent

The tumour-producing activity of cell-free extracts of this sarcoma can be removed by high-speed centrifugation and the agent can be recovered quantitatively from the deposit so obtained. When examined under the dark-field microscope such deposits are found to contain large numbers of particles, many of which have the optical properties (high degree of apparent luminosity and uniformity of apparent size) which are characteristic of virus bodies. By repeated fractional centrifugation it is possible to obtain relatively pure suspensions of the bodies. These show a high degree of tumour-producing activity and, in addition, are specifically agglutinated by the sera of fowls in which a des Ligneris tumour has regressed. These findings indicate that the active agent of this fowl tumour is similar in nature to the extrinsic agents of the Rous No. 1 and Fujinami sarcomata, the particulate nature of which has been demonstrated by several independent investigators (Ledingham and Gye, 1935; McIntosh, 1935; Amies, 1937; Claude, 1937). For the sake of brevity, protocols of the experiments on which these statements are based will be omitted, but examples of the activity of deposits obtained by high-speed centrifugation will be found in the experiments which follow.

Immunological Experiments

It is well known that antibodies capable of neutralising the tumour agent *in vitro* are developed in the sera of fowls in which a filterable tumour is growing or has recently regressed. This property has been utilised by Andrewes (1931–1933) and others to determine the relationships of these fowl tumours. Andrewes concluded that all the tumours which had been thoroughly tested had some degree of antigenic relationship, but that no two were serologically identical. Des Ligneris reported that the sera of fowls bearing the new tumour did not show inhibitory properties towards the Rous No. 1 or the Fujinami agents, and that, conversely, sera of fowls bearing Rous No. 1 or Fujinami tumours did not inhibit the filterable agent of the new tumour. It was on this evidence that he concluded that the new tumour was a distinct entity.

(*A*) *Cross-infection Experiments:* A number of fowls in which a des Ligneris tumour had developed and subsequently regressed were inoculated

with Rous No. 1 agent in the form of a cell-free tumour extract or a deposit obtained from such by high-speed centrifugation. These tumour agent suspensions were titrated at the same time on groups of normal chickens to determine their activity. Among 14 des Ligneris-immune fowls so treated 7 developed tumours as the result of the subsequent inoculation of Rous No. 1 agent. These were small, slowly growing tumours produced by the inoculation of 200 to 400 minimal tumour-producing doses of the extract or suspension. The 7 fowls which failed to develop tumours had received 40 to 400 minimal doses. The converse experiment of inoculating the des Ligneris agent into Rous-immune fowls yielded a very different result. Large rapidly growing sarcomata developed as the result of injecting des Ligneris agent in each of the 13 fowls that were tested. The dosage varied among individual birds from 400 to 4000 minimal tumour-producing doses and was thus somewhat larger than that given to the first group. Rous-immune fowls are thus fully susceptible to the des Ligneris agent, whereas des Ligneris-immune fowls frequently possess a complete or partial immunity to the Rous agent. Andrewes (1933), working with immune duck sera, was able to demonstrate a similarly one-sided relationship between the Rous No. 1 and the Fujinami tumour agents. The present experiments provide a clear demonstration that the Rous No. 1 and the des Ligneris tumours are not identical, since fowls in which a Rous sarcoma has developed and subsequently regressed are solidly immune to re-inoculation with the same tumour agent.

(*B*) *Neutralising Antibodies in Sera of Rous-immune and des Ligneris-immune Fowls:* Little importance can be attached to the bare statement that a given serum inhibited or failed to inhibit the activity of a certain tumour agent. Only a quantitative experiment is capable of giving reliable information on a problem such as the present one, in which the identity of two filterable agents is being investigated. Owing to the lability of the tumour agents it is impossible to carry out a preliminary titration of the extract or suspension which is to be used for *in vitro* neutralisation tests. This is a serious disadvantage because it prevents the experimenter from choosing the proportions of agent and serum which are to be allowed to interact. It is possible, however, to obtain quantitative data by titrating the activity of the tumour agent suspension simultaneously on a separate group of chickens and by testing two or more dilutions of the serum against a constant amount of agent in the neutralisation test itself. This plan was adopted in the following experiments.

A number of fowls in which des Ligneris tumours had developed and subsequently regressed were given repeated injections of the same tumour agent in order to render them strongly immune. By the same method a second group of fowls was rendered highly resistant to the Rous No. 1 agent. The serum of each bird was then tested for neutralising antibodies against both tumour agents. Mixtures of the serum and tumour agent were prepared, incubated at 37° C. for one hour, and then inoculated either immediately or after storage overnight at 4° C. into groups of normal chickens. Control mixtures of normal serum with the same amount of tumour agent suspension were treated in the same manner and inoculated in a corresponding site (breast muscle or leg) on the opposite side of the same fowls. Protocols of two of the six experiments which were carried out are given in Table II. The results

TABLE II: *Cross-neutralization Experiments*

(A) Rous Agent Neutralised by Des Ligneris-Immune Serum

Site	Inoculum *	Fowl 165	Fowl 166	Fowl 167
R. breast	Rous T.A.S. + undiluted serum of des Ligneris-immune fowl	Nil	Nil	Nil
L. breast	Rous T.A.S. + undiluted normal fowl serum	++	++	++
R. leg	Rous T.A.S. + serum of des Ligneris-immune fowl diluted 1 in 5	Nil	Nil	Nil
L. leg	Rous T.A.S. + normal fowl serum diluted 1 in 5	+++	++++	++++
		Fowl 168	Fowl 169	Fowl 170
R. breast	Rous T.A.S. + serum of des Ligneris-immune fowl diluted 1 in 20	++	+	+
L. breast	Rous T.A.S. + normal fowl serum diluted 1 in 20	++++	++++	++++
R. leg	Rous T.A.S. diluted 1 in 25	++	++	+++
L. leg	Rous T.A.S. diluted 1 in 125	+	Nil	Nil

(B) Des Ligneris Agent Neutralised by Rous-Immune Serum

Site	Inoculum	Fowl 342	Fowl 343	Fowl 344
R. breast	Des Ligneris T.A.S. + serum of Rous-immune fowl diluted 1 in 5	++	+	+
L. breast	Des Ligneris T.A.S. + normal fowl serum diluted 1 in 5	++++	+++	++
R. leg	Des Ligneris T.A.S. + serum of Rous-immune fowl diluted 1 in 25	+++	++	++
L. leg	Des Ligneris T.A.S. + normal fowl serum diluted 1 in 25	++++	+++	++
		Fowl 345	Fowl 346	Fowl 347
R. breast	Des Ligneris T.A.S. + serum of Rous-immune fowl diluted 1 in 125	+++	*	++
L. breast	Des Ligneris T.A.S. + normal fowl serum diluted 1 in 125	+++	*	+++
R. leg	Des Ligneris T.A.S. diluted 1 in 25	++	Nil	+
L. leg	Des Ligneris T.A.S. diluted 1 in 125	+	Nil	+

T.A.S. = tumour agent suspension. The number of plus signs indicates the size of the tumour found at the *post-mortem* examination.

* Small tumours developed and subsequently regressed.

show quite clearly that these two tumours cannot be differentiated by cross-neutralisation tests. Similar evidence was obtained from the four remaining experiments, but the results in these were less definite owing to the low antibody titres of the sera employed.

Experiments have also been carried out with the sera of rabbits which had been repeatedly inoculated with tumour agent suspensions prepared from Rous

No. 1 and des Ligneris sarcomata. Neutralisation tests with these antisera demonstrated that each tumour agent is inhibited by the homologous and, to a lesser degree, the heterologous serum. These experiments will be described fully in a subsequent publication.

Experiments on Ducks

The results so far obtained seemed to indicate that the des Ligneris tumour was similar to the Fujinami sarcoma in many of its biological characters. It was therefore determined to test the susceptibility of ducks to the des Ligneris agent. Owing to restrictions of space it was not possible to do this at the Lister Institute and the investigation was therefore carried out at the National Institute for Medical Research by Dr. W. J. Purdy, who kindly supplied the following notes on the results he obtained.

Cell transplantations of the des Ligneris tumour into Khaki Campbell ducks follow the same course as grafts of Fujinami sarcoma. They grow at about the same rate and, if they do not kill the bird, they tend to regress at about the same time. Transplantation from duck to duck is no more difficult in the case of one tumour than in the other.

A clear pulp-filtrate of fowl-grown des Ligneris tumour readily produced tumours when injected into ducklings which had reached such an age that one could no longer expect even cells of a Rous No. 1 sarcoma to grow in them. These filtrate tumours which grew in ducklings appeared earlier and grew more rapidly than those produced in fowls by the same filtrate, just as occurs in the case of the Fujinami sarcoma. This same clear and evidently cell-free filtrate produced tumour nodules on the chorioallantoic membrane of duck embryos. Microscopically, these nodules showed the structure of a spindle-cell sarcoma.

Naked eye examination of tissue from examples of duck-grown des Ligneris tumour revealed nothing which was obviously different from what is commonly found in duck-grown Fujinami tumours. On examination five or six days after inoculation, while growth is still rapid, both tumours show the same range of variation in structure. Sometimes, for example, one may find a tumour consisting only of highly congested, even haemorrhagic, tissue; sometimes only of soft, white, translucent tissue; and often of a mixture of these two types.

There is evidence that ducks which have recovered from a des Ligneris tumour are then resistant to the Fujinami sarcoma. Seven birds which had recovered from des Ligneris tumours induced by infective material obtained from fowls, and 2 ducks in which similar material had failed to produce tumours, each received a test dose of duck-grown Fujinami extract. At the same time 10 normal ducks of similar ages received an equal volume of the same filtrate. This filtrate proved to be of rather low infectivity. No tumours developed in the ducks in which des Ligneris tumours had regressed; but of the 10 ducks used as controls, 4 developed unmistakable tumours, 2 developed what appeared on dissection to be small tumours, and the remaining 4 failed to develop tumours. The results obtained in this experiment cannot be attributed to the development of anti-fowl bodies as the result of the first

inoculation since the Fujinami filtrate which was subsequently injected was derived from a duck-grown and not a fowl-grown tumour.

Conclusions

Before entering into a discussion of the results it is necessary to state that no experiments with the Fujinami sarcoma have been undertaken in this laboratory during the last two years. The possibility that the materials used in this investigation were accidentally contaminated with this tumour or its filterable agent can therefore be excluded with certainty. It was in order to avoid this criticism that we have omitted certain obvious experiments such as the determination of the susceptibility of des Ligneris-immune fowls to the Fujinami tumour agent.

With the methods at present available it is probably impossible to produce formal evidence of the identity of two avian tumour agents. The most that can be achieved is to demonstrate by a study of the general characteristics of the tumours and of their immunological relationships that the two behave in a similar manner. The same problem arose in the case of the differentiation of chicken tumour nos. I and XLIII of the Rockefeller Institute (Lange, 1914).

Apart from the peculiar circumstances of its origin, the des Ligneris sarcoma appears to possess no characteristics which differentiate it from the other filterable fowl tumours. Furthermore, the finding that cell-free extracts of this tumour are capable of producing progressive growth in ducklings immediately places it in close relationship with the Fujinami sarcoma. Other facts demonstrated during this investigation provide additional support for this view. In its structure and behaviour the new tumour closely resembles the Fujinami sarcoma and its immunological relationship with the Rous No. 1 sarcoma is compatible with the same conclusion. The statement that the sarcomata of Fujinami and des Ligneris are indistinguishable from each other seems, therefore, justifiable.

The main interest lies, of course, in the manner in which the des Ligneris tumour originated. If the possibility of an accidental contamination of the tissue cultures can be excluded (and we do not question Dr. des Ligneris' assurances on this point), then a repetition of the original experiments, preferably in a laboratory where no other work on filterable fowl tumours is being done, is clearly indicated.

Summary

(1) A study has been made of the des Ligneris sarcoma—a filterable avian tumour which originated in a fowl inoculated with normal chicken fibroblasts which had been cultivated *in vitro* with dibenzanthracene.

(2) In its structure and behaviour this tumour closely resembles the Fujinami sarcoma. This view is considerably strengthened by the fact that cell-free extracts give rise to progressive tumours in ducklings. Ducks in which a des Ligneris tumour has developed and subsequently regressed are immune to the tumour agent of the Fujinami sarcoma.

(3) Various immunological experiments indicating that the des Ligneris tumour is related to Rous sarcoma No. 1 are compatible with this belief.

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OBSERVATIONS UPON SPONTANEOUSLY RECURRING ROUS NO. 1 TUMOURS.

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THE usual result of the inoculation of Rous No. 1 agent into a fowl is the production of a rapidly-growing, very malignant sarcoma which kills the bird in 3–4 weeks. Several workers have described cases in which the tumours do not grow so rapidly, may remain more or less stationary in size for a considerable period, or regress completely. The general impression is that this is more likely to occur in older birds, and it has been shown that many fowls spontaneously develop antibodies to the Rous No. 1 agent as they grow older (Ledingham and Gye, 1935; Amies, 1937; Duran-Reynals, 1940). Since tumour growth is slower in old birds, this is regarded as evidence of reduced susceptibility.

It has recently been found at this Institute that the suceptibility of healthy 6-week-old Brown Leghorns to inoculation with the Rous No. 1 sarcoma is chiefly determined by the genetic make-up of the individual, and it has been found possible to breed a strain of birds, known as the "Non-Susceptible" or "N-S" strain, which are very resistant to inoculation with the Rous No. 1 agent. The study of this strain is hoped to lead to valuable information as to the mechanics of resistance and susceptibility to neoplasms. An unexpected finding during this work was that Rous tumours appeared in many birds a long time after the inoculation of agent, the original induced tumour (if any) having vanished a considerable time previously. Most workers cannot, for lack of accommodation, keep fowls under observation for extended periods, so that this phenomenon is not likely to have been noticed.

To date 15 instances of recurring tumours have been noted, of which 13 were found in the "N-S" strain. There could be no doubt that these were connected with the inoculation of Rous agent, for spontaneous tumours are very rare in the Institute flock (less than 1 per year). The exact incidence of recurring tumours cannot be stated with any accuracy because of the fluctuating nature of the population, but it is certainly over 10 per cent. The N-S strain are all tested at the age of about 6 weeks by inoculating a dose of Rous agent into the right breast, and successive decimal dilutions of this into the left breast, right leg and left leg respectively. The activity of the agent suspension is simultaneously determined by inoculation in a similar manner

into a group of control birds, and is usually 50–500 minimal infective doses (M.I.Ds.). Most N-S birds grow small tumours at the sites given the largest doses, and these later regress in about 75 per cent. of cases within a period of 28 days. The longest interval found between this test inoculation and the appearance of the delayed tumour is over one year. This case is quoted as an example of the type of finding. Others illustrating various aspects are quoted later.

Fowl L.1833, tested 7.vi.39, dose approximately 1000 M.I.Ds. No tumours detected over a period of 5 weeks. Used for breeding. First egg 18.xi.39, laying and fertility normal, last egg 3.vi.40. Found dead 12.vi.40. At autopsy, typical rapidly-growing Rous sarcomas were found, the largest almost filling the right breast, and successively smaller ones occurring in the left breast and right leg. No tumours were found at any other sites.

In none of these birds have tumours been found at sites other than those inoculated by agent. Though it is extremely unlikely that these tumours could be anything other than Rous No. 1 tumours, it was considered desirable to determine whether or not they contained any Rous agent. The demonstration of agent proved a matter of some difficulty, as the tumours that were discovered by occasional inspection were mostly of the slowly-growing type, from which only a small amount of agent could be anticipated. The rapid tumours usually killed their host before discovery, and were not very common. In 5 cases attempts were made to transmit the tumour, details of which are given later. In these experiments the agent was always prepared by the method of Amies and Carr (1939) (centrifuging a cell-free extract at pH5 on a laboratory centrifuge). Glycerol was used in many cases as a preservative and to ensure that no cells were transmitted. Since there are few data on the rate of loss of activity of Rous agent in 50 per cent. glycerol, the following experiments are submitted in justification of its use in the present work. They are the first three experiments in which the preserving activity of glycerol was quantitatively investigated. The amount of agent in tumours in this paper is given as the least amount of tumour which would yield 1 M.I.D. of agent, as determined by titrating the final product of the processing into 6-week-old susceptible chicks. The activity of a rapidly growing tumour in a young bird lies between 10^{-5} and 10^{-6} g.

EXPERIMENTS ON THE PRESERVING ACTION OF 50 PER CENT. GLYCEROL.

Experiment 1. *Tumour of Fowl* 168.

Processed fresh 2.iv.41. Activity in 2×10^{-5} g. at least.
Part of the tumour preserved in glycerol and processed 14.v.41. Activity in 5×10^{-5} g. at least.

Experiment 2. *Tumour of Fowl* 184.

Processed fresh 26.v.41. Activity in 10^{-6} g.
Part of the tumour preserved in glycerol.
A part of this processed 10.vi.41. Activity in 5×10^{-6} g. at least.
Rest processed 18.vi.41. Activity in 10^{-4} g.

Experiment 3. *Tumour of Fowl* 259.

5.viii.41 : Processed fresh, and agent preserved in 50 per cent. glycerol.
13.viii.41 : Activity corresponded to 5×10^{-6} g. tumour at least.
29.viii.41 : Activity corresponded to 5×10^{-5} g. tumour.
15.ix.41 : Activity corresponded to $2{\cdot}5 \times 10^{-4}$ g. tumour.

It may therefore be assumed that agent could be detected in tumour or suspension preserved by this method if the original tumour contained any recognizable amount of agent. The method of processing enables the final concentration of agent per c.c. to reach any desired value. The usual figure chosen was such that the largest amount in the titration was the equivalent of about 10^{-2} g. tumour. A negative result then indicates that the amount of agent is less than 1/1000 of the amount expected in a routine tumour.

EXPERIMENTS ON THE AGENT CONTENT OF THE RECURRING TUMOURS.

Experiment 1. *Fowl* 0.620.

30.v.41 : Tested, the largest dose containing about 100 M.I.Ds. Small tumours appeared, rapidly regressing.
1.viii.41 : Last inspection before tumour appeared.
20.ix.41 : The right breast was found to be filled with a soft tumour, from the centre of which fluid was frequently tapped.
2.x.41 : Some of this fluid was mixed with an equal volume of glycerol and placed in the cold. Next day it was diluted with 4 volumes of saline and 0·5 c.c. injected into 3 birds. No tumours resulted.
12.x.41 : The bird was found dead. Some of the tumour was placed in 50 per cent. glycerol, and processed for agent 14.x.41. The product was injected in amounts equal to 5×10^{-2} g. into 3 birds. No tumours resulted.
Result.—No agent demonstrated. The type of material used was not particularly suited for its detection.

It has been shown by Andrewes (1931) that tumour exudate may possess strong powers of inactivation towards tumour agents.

Experiment 2. Exact identity of bird unknown, as it was one of two birds in a pen which had lost their identifying wing tabs. It was known to have been tested before 6.v.41.
5.xi.41 : Large tumour found in right breast. The bird was killed, and the tumour found to be white, fibrous, and with a caseous centre. A part of this was transplanted as cells to birds 383, 384, and 385, and gave rise to rapid tumours in all three birds. Another part was preserved in glycerol, and a part immediately processed for agent. This was inoculated into 3 birds at a dose corresponding to 10^{-1} g. tumour. No tumours resulted.
10.xi.41 : The tumour in glycerol was processed for agent, and a dose corresponding to 5×10^{-2} g. tumour inoculated into 3 birds. No tumours resulted. The tumours of birds 384 and 385 were processed for agent, and the yield found by titration to be 10^6 and 10^5 M.I.Ds. per gramme of tumour respectively.
Result.—Agent not present in detectable amounts in the original tumour, but present in normal quantities in transplants.

Experiment 3. *Fowl* 0.274.

6.v.41 : Tested, the largest dose containing about 1000 M.I.Ds. Small tumours appeared in the breasts, but soon regressed.
16.ix.41 : Tumours found in breasts, that on the right being estimated as containing 15 g. material, that on the left less than 0·5 g. Both tumours were found to decrease in size during the next few days.

28.xi.41 : Most of the tumour remaining in the right breast was removed by operation under ether anaesthesia. Two birds were implanted with cells of this material. No tumours resulted. A portion of the tumour was kept in glycerol, and processed for agent 1.xii.41. Amounts corresponding to 2×10^{-2} g. tumour inoculated into 3 birds failed to give rise to any tumours.

29.xi.41 : First egg laid. Further eggs 1.xii and 3.xii.

3.xii.41 : The bird was found to have a severely lacerated comb. This healed easily on treatment, but she was very anaemic and laying ceased. The small tumour in the left breast now began to grow rapidly, and a part of this was removed at biopsy 16.xii.41. Implants into 3 birds failed to produce tumours.

The tumour began to decrease in size again about 10.ii.42 ; laying began again 20.ii.42, and the tumour soon disappeared and has not returned.

Result.—No agent could be demonstrated.

Experiment 4. *Fowl* 0.106.

2.iv.41 : Tested, the largest dose containing about 1000 M.I.Ds. Very small tumours appeared and soon regressed.

16.ix.41 : Tumours found in breasts, that in the right estimated at about 20 g., that in the left about 6 g.

19.ix.41 : First egg laid, followed by 2 more, and then the bird began a partial moult, and the tumours began to decrease in size.

4.xii.41 : Some of the tumour of the right breast was removed under ether. The tumour was very tough. Some was transplanted into 2 birds, but no growth resulted. A portion was preserved in glycerol, and processed next day. Amounts equivalent to $2{\cdot}5 \times 10^{-1}$ g. tumour failed to give rise to any tumours in three birds.

18.xii.41 : The tumour in the left breast had begun to grow very rapidly after the operation, and a portion of this was now removed. This tumour was found to be quite soft. Transplantation of this into 3 birds resulted in all developing very rapid sarcomas. A part of the tumour was processed and the agent preserved in glycerol. This was later injected into 3 birds in amounts corresponding to $2{\cdot}5 \times 10^{-2}$ g. tumour. No tumours resulted. The tumour of one of the transplanted birds was found to contain at least 10^5 M.I.Ds. per gramme of tumour.

3.i.42 : The bird was killed with a large tumour filling the left breast, a tumour half this size in the right breast, and a small tumour in the left leg.

Result.—No agent detected in original tumour, but found in usual amounts in transplants.

Experiment 5. *Fowl* 0.1422.

25.x.41 : Tested, the largest dose containing about 100 M.I.Ds. A single small tumour appeared in the right breast, and rapidly regressed.

26.i.42 : Injected 1·5 mg. methylcholanthrene dissolved in arachis oil into left leg.

7.iii.42 : Injected 5 mg. methylcholanthrene in same way. Similar treatment was given to 4 other N-S birds of about the same age, without the appearance of recurring tumours.

14.iv.42 : Small tumour noted in right breast, and this rapidly enlarged, and by 28.iv.42 filled the whole breast. The bird was then killed, and the tumour processed, the product being inoculated into 2 chicks in amounts equivalent to 10^{-1} g. tumour. No tumours resulted.

Result.—No agent demonstrated.

A portion of all the tumours removed from the last four birds with recurring tumours and all tumours resulting from cell implants or inoculation with agent were examined histologically. In all cases the appearance was that of a Rous No. 1 sarcoma. When a negative result followed inoculation of cells or agent preparation from these recurring tumours into other birds, one or more of the group was later inoculated with corresponding material from a routine Rous tumour. In all cases the birds were thus proved to have been susceptible.

It thus appears that the transplants of the recurring tumours are typical in every way. One line of tumour originating in this way from Expt. 2 was carried for three further generations by agent, and was indistinguishable from the routine Rous tumour in every respect. The apparent absence of the agent in the original recurring tumours is notable. The fact that in most cases the tumour was growing rather slowly and in an old bird may partially explain the failure to find any agent, but the point seems to call for further investigation.

TRANSPLANTABILITY OF THE RECURRING TUMOURS IN N-S BIRDS.

These recurring tumours may have appeared either as a result of a decrease in the susceptibility of the host, or as the result of the mutation of either a tumour cell or an agent particle to a form which would grow more readily in the host. The absence of detectable agent, and the appearance of the tumour at more than one inoculation site, do not favour the latter hypothesis. If such a change had occurred in the tumour, it is reasonable to expect that the tumour would grow also in ordinary N-S birds. Attempts to obtain growth of either the cells of the recurring tumours, or grafts of them growing in normal chicks, or of agent preparations of either of these, by inoculating them into N-S birds whose agent-induced tumours had regressed, were never successful.

The alteration which elicits the formation of the tumour is therefore presumably in the host and not in the tumour.

EFFECT OF LAYING ON THE GROWTH OF RECURRING TUMOURS.

The suggestion in the case of 0.274, in Expt. 3, that egg production and tumour growth are antagonistic has been noticed in several instances. Another case may be briefly mentioned as having some interesting features.

Bird 0.135. 22.iv.41. Tested, largest dose containing about 50 M.I.Ds. Small tumours soon regressed. Laying 7.iii.41 to 3.x.41, then stopped. Small tumour found in right breast 20.xii.41. Laying began again 1.i.42, and by 10.i.42 no tumour was detectable.

20.i.42 : Comb was injured by bird in neighbouring cage. Laying ceased, and the tumour began to grow again. This growth never reached a large size, and later regressed. Laying recommenced on 23.ii.42, and the bird has remained normal.

This is not an invariable finding, however, for there have been two cases in which the bird has continued to lay in spite of a recurring tumour found at a periodic inspection.

TRAUMA IN RELATION TO INDUCTION OF RECURRING TUMOURS.

As will be noted in the case histories quoted, there are occasions in which the recurring tumour is prefaced by a history of injury, due either to accident or operation. There are two other examples in the present series. One may be briefly mentioned.

Bird 0.311. Tested 27.v.41, the largest dose containing about 1000 M.I.Ds. 1.vii.41 : Skin on top of head badly torn, presumably by the other birds in the pen. Healing was rapid, but tumours were seen to be developing a few days later, and the bird was killed 8.viii.41, and found to have several nodules of tumour in the right breast, nothing in the left breast, a very large tumour in the right leg, and nothing in the left leg. There was no indication of tumour formation at the site of the head injury.

Stimulation of tumour growth as a result of injury has not been observed in young birds. On several occasions the N-S birds have been injured by their cage-mates while being tested, but this has never resulted in any increased growth of tumour. Also, there have been some cases of accidental injury to mature N-S birds without any tumour growth resulting. This connection between tumour recurrence and injury is therefore no more than suggestive.

SEX INCIDENCE IN N-S BIRDS.

All 13 cases of recurring tumours in the N-S strain have been found in females. No significance can be attached to this, however, as only a very few males are retained for breeding purposes.

BIRDS OTHER THAN N-S STRAINS.

There have been two other examples of this delayed growth of tumours in birds from other strains of the Institute stock. One was a male which had grown very small tumours which soon regressed, and the other was a male which produced no tumours at all in response to a large dose of agent. Each bird was found with large progressive tumours about two months after inoculation.

DISCUSSION.

The frequency with which this recurrence has been found is certainly surprising. It appears to occur in birds at about the time that the early rapid growth is becoming reduced, and usually also in non-layers. As suggested above, the cause is most likely to be a decrease in the resistance of the host. Such a decrease of resistance to tumour growth with age is contrary to many of the findings of the workers who use mammalian transplanted tumours. But the two cases are not at all comparable, since the conditions governing tumour incidence and host resistance in this work are quite different from those encountered in most other types of experimental tumour research. Although many experimenters have shown (Ledingham, 1935 ; Amies, 1937 ; Duran-Reynals, 1940) that old birds develop serum antibodies to Rous agent as they age, and that tumours grow more slowly in older birds, neither of these facts are proofs of increased resistance, for such antibodies have no effect upon the growth of tumour cells, and the reduced rate of growth may well be due merely to the reduced rate of vital processes in old animals.

It was possible to test this hypothesis on a limited scale by re-inoculating with Rous agent 4 tested N-S males one year after the test inoculation. In all birds the tumours were found to reach a much larger relative size and to regress

very much more slowly than at the first test. In one case the tumour grew until it killed the host. The tumours certainly grew rather more slowly than in young chicks, but the fact that any growth at all took place in animals which had received the equivalent of an immunizing dose indicated that a marked reduction in resistance had indeed occurred.

The chief objection to such a theory lies in the fact that only a proportion of the birds developed the recurring tumours, whereas according to this supposition, the resistance of all birds will decrease with age. Either in some birds the cell or agent responsible for the tumour did not survive at all, or the degree to which the resistance drops is not sufficient for tumour growth to occur in many birds. A variation in this decrease of resistance is certainly indicated by the variation in the time between inoculation and the development of the recurring tumour. It may also be that some stimulus is required in addition to provoke tumour development. The relation of trauma to the development of the tumours makes it possible that this may act either by providing a direct stimulation for tumour growth, or by reducing the resistance of the host to tumour development. Such a stimulation of a latent tumour bears a close resemblance to the findings of MacKenzie and Rous (1941) on the effect of wounds on tar lesions in rabbits. It is notable that all the recurring tumours (except the methylcholanthrene-treated 0.1422) were found in birds running together in a pen, where trivial injuries due to fighting, etc., are common, and never in those birds kept in individual cages, where such injuries are rare. Such stimulation as a result of injury is confined to the older birds, however.

Since the tumours appeared only at the site of injection, it must be presumed that tumour cells or agent were immobilized and preserved at these points. The absence of tumours at other points suggests that any agent which may have been disseminated during the growth of the first tumour (Mellanby, 1938) either was not thus preserved, or was no more capable of inducing a tumour at a later time than at the beginning. It is difficult to imagine that the agent which remained at the site of injection could have any other than an intracellular existence. If this cellular infection produced a neoplasm, as one would expect, it must have been exceedingly small in size, for post-mortem examination of a large number of birds whose tumours had regressed has never revealed anything which could be regarded as a latent tumour ; the regression has always been complete, as far as could be judged by macroscopical examination. In addition, since the relative sizes of multiple tumours often approximated to the size expected from a consideration of the dose of agent originally given, more than accidental survival of a single agent particle or cell is involved, and it becomes even more difficult to obtain a clear idea of the conditions which govern such a survival.

A further consideration of this matter is best left until some data as to the cause of the resistance of these birds to the inoculation of Rous agent becomes available. It is to be hoped that this will also shed some light on the mysterious apparent absence of the agent in these tumours, and the equally strange reappearance of it in cell grafts into other birds. Though the sudden appearance of a non-filterable phase of Rous tumour is well recognized, this has usually been of a capricious type. In the present work this state of affairs

regularly follows a certain procedure. The failure of the agent preparations to produce tumours cannot be due to the presence of inhibitory substances of the type which has often been reported in filtrates of the Rous No. 1 sarcoma by several workers (Gye and Purdy, 1931 ; Sittenfield, Johnson and Jobling, 1931 ; Murphy and Sturm, 1932), since the agent was always separated from the extract by the method of processing which was here employed, and the inhibitor should have remained behind. The only factor which differs from the normal seems to be the age of the birds. Unfortunately present conditions make it impossible to carry out any work requiring a number of old birds, so that both this suggestion, and the suggestion that older birds are more susceptible, cannot be directly tested.

Finally there can be no doubt that our ideas as to the range of action of the classical tumour-inducing viruses must be considerably extended.

SUMMARY.

A number of cases are described of Rous No. 1 tumours appearing many months after inoculation of the agent into birds and regression of any tumours thus induced. This is believed to be due to a reduction in the resistance of the host. Agent could not be demonstrated in such tumours, but was present in the usual amounts in grafts from them.

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THE EFFECT OF SOME SUBSTANCES INFLUENCING CELL ACTIVITY UPON THE GROWTH OF THE ROUS No. 1 SARCOMA.

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A LARGE number of experiments have been reported describing the effect of the administration of substances upon malignant growths. Few workers have utilized the virus-induced avian sarcomata for this type of work, yet in many respects these tumours are the most satisfactory material for such investigations. The Rous tumour originates from the animal's own tissues, and hence iso-antibody effects are eliminated, and it is unlike spontaneous or chemically-induced neoplasms in that the biological and chemical nature of the Rous tumour is the same in each animal. At the end of experiments designed previously to determine the mode of inheritance of the resistance of fowls to inoculation with the Rous No. 1 agent, a number of birds with small tumours whose further progress was very slow were available. These seemed suitable for testing the effect upon the Rous No. 1 sarcoma of various substances which from their known action on cell activity might be expected to have some effect upon the tumour, and the results are reported in this paper.

MATERIAL.

Except where otherwise stated, all birds were from the "Non-Susceptible" strain of the inbred Brown Leghorns maintained at this Institute. At the age of 6 weeks they had been inoculated with the Rous No. 1 agent, the largest dose of about 1000 minimal infective doses being given into the muscles of the right breast, and decimal dilutions of this into the opposite breast, right and left leg respectively. Even the largest doses produced only small tumours, about 0·1 to 5 g. weight. The test was terminated 28 days after inoculation, by which time the tumours had been about the same size for 10 days. Untreated birds showed slow growth or a slow regression of the tumour during the next 14 days. As such changes are not subject to irregular fluctuations, the birds were used within a week of the end of the susceptibility test.

Other tests were performed upon birds from other strains of the Institute Brown Leghorns taken at random, and these will be referred to as "controls."

EXPERIMENTAL.

Experiments with colchicine.

The ability of colchicine to arrest mitosis has led several workers to try its action upon neoplastic growth. An inhibition of the development of the Shope papilloma as a result of administering colchicine has been reported by Peyron and collaborators (1936, 1937). No effect was found on tumours of rats and mice by Brues, Marble and Jackson (1940), even when sublethal doses were employed, and a similar lack of effect is reported by Clearkin (1937), Ludford (1936) and by Poulsson (1935) who used mouse tumours alone. These negative results were obtained with both spontaneous and transplanted tumours. Amoroso (1935) in a preliminary paper stated that regression of mouse tumours (M.63) was obtained after treatment, and Dittmar (1939) claimed a reduction in growth of the Ehrlich mouse sarcoma, while Lits (1936) and Lits, Kirschbaum and Strong (1938) stated that mice bearing a lymphoid leukaemia survived longer if treated with colchicine.

Solutions containing 1 mg. of colchicine were injected into 11 fowls bearing Rous tumours. One bird died, and most of the rest were unwell for about 24 hours, but recovered soon after. Doses of 2 mg. were invariably fatal. In seven of the birds the colchicine had no apparent effect upon the tumour, and in the remaining three, a possible slight stimulation of the tumour was recorded. Microscopical examination of the testes of birds given this amount of colchicine 24 hours previously showed an undoubted arrest of cell division. Administration of this amount of colchicine to birds whose tumours had completely regressed a short time previously did not produce any recurrence of the tumour. The same amount given to four "control" birds with small, non-progressive tumours produced in all cases an apparent stimulation of the tumour.

In spite of the failure of colchicine to arrest the growth of the tumour, an inactivation of some of the virus may have occurred. To test this, 1 mg. of colchicine was injected intravenously into a "control" fowl bearing large tumours produced by an inoculation of agent 29 days previously. The bird reacted badly to the injection, but had almost completely recovered by the next day. It was then killed, and a portion of the largest tumour (which was very haemorrhagic) was processed to recover the virus by the method described by Amies and Carr (1939) (centrifuging cell-free extract at pH 5 on a laboratory centrifuge). Tumours were produced by an amount of this processed material corresponding to 10^{-5} g. of tumour, which is within the limits usually found in the Rous No. 1 tumour (10^{-5} to 10^{-6} g.). No inactivation was therefore demonstrated. In addition, the effect of colchicine *in vitro* upon the Rous No. 1 agent freed from cells was determined. A suspension of concentrated virus was diluted until the amount present per c.c. was equal to the yield from 1 g. of tumour, and two lots of 1 c.c. taken. To one, 0·1 c.c. of a solution of colchicine containing 0·375 mg./c.c. was added, and half an hour later the two solutions were titrated simultaneously upon two groups of three birds, with the result shown in Table I. It is evident that no permanent inactivation of the virus had occurred.

TABLE I.—*Effect of Colchicine on Free Rous Agent.*

Dilution.	Site.	Fowl 212. Control treated.		Fowl 213. Control treated.		Fowl 214. Control treated.	
10^{-3}	Breasts	+	+	+	+	+	+
10^{-5}	Legs	+	+	+	+	+	+
		Fowl 215.		Fowl 216.		Fowl 217.	
10^{-4}	Breasts	+	+	+	+	+	+
10^{-6}	Legs	−	−	+	−	−	+

+ Indicates tumour growth. The size of the tumour on each side was substantially the same.

Finally, three " control " birds were injected with colchicine and later inoculated with Rous No. 1 agent, in order to see if the susceptibility of the cells to the agent was affected by this treatment. After injection of 1 mg. of colchicine, one died within 24 hours, and the others were inoculated with decimal dilutions of a suspension of Rous No. 1 agent of rather weak activity the day after colchicine was given, and three untreated " control " birds were similarly injected with the agent. Tumours grew equally well in all birds.

In addition five birds bearing Rous sarcomas were given colchicine and then killed 1–5 days later, and the tumour examined histologically. An increase in the amount of haemorrhage and leucocytic infiltration was found, and this may have been responsible for at least part of the increase in tumour size sometimes noted after treatment.

Experiments with acenaphthene.

It has been shown (Kostoff, 1937, 1938 ; Levan, 1940) that acenaphthene has an action rather similar to that of colchicine, and is active in certain cases (e.g., in *Colchicum* (Levan)) where colchicine has no effect. Though it is present in the carcinogenic fraction of tar, it is devoid of carcinogenic properties when tested upon mice (Bloch, 1922 ; Twort and Fulton, 1930 ; Kennaway, 1930). Haddow and Robinson (1937) reported that it would slightly inhibit the Walker rat carcinoma 256, and found that it produced inhibition in one out of five spontaneous mouse neoplasms.

Since acenaphthene is only feebly soluble in water, it was dissolved in arachis oil (1 g. in 10 c.c.) by heating in a water-bath, and after cooling in air, 0·5 to 1 c.c. of the resulting fine suspension was injected into the left leg of 24 birds bearing tumours. A fine suspension is necessary in order to produce activity in plants (Kostoff, 1938). The oil alone produced no effect when tested on three tumour-bearing birds. The results were very irregular, seven birds showing no effect, ten showing a regression of the tumours, and seven showed a more or less marked stimulation of the tumours. Some of the birds showing regression were kept for several months, but there was no reappearance of the tumour. In four of the cases in which the tumour was stimulated the largest tumour was not acted upon to an extent which enabled it to maintain its superior size.

Case history. Fowl G.558.—Inoculated with Rous No. 1 agent 10.vi.41.

Small tumours appeared in breasts only, and these remained small until 10.vii.41. (Three controls injected with the same material had produced tumours with all dilutions of the agent in two cases, and with the three highest concentrations only in the other bird.) The bird was then given 1 c.c. of the acenaphthene solution into the left leg (site of inoculation of the highest dilution of agent). The tumours began to grow rapidly, and post-mortem on 22.vii.41 the conditions were as shown in Table II.

TABLE II.—*Effect of Acenaphthene on Tumours.*

Agent given 10.vi.41.	Site.	Tumour size. 10.vii.41.	Tumour size. Post-mortem 22.vii.41.
Full dose (100–1000 min. infective doses)	R. breast	±	Doubtful trace
1/10 full dose	L. ,,	±	++
1/100 ,,	R. leg	—	++++
1/1000 ,,	L. ,,	—	—

The range of tumour size from least amount visible to maximum is expressed by the series ±, +, ++, +++, ++++.

This type of result appeared irregularly, and neither parentage nor original tumour size seemed to be concerned. The cells of one of these stimulated tumours was transplanted to five " non-susceptible " birds whose tumours had regressed, but growth did not result in three birds, and only a very small tumour appeared in the others. This is similar to the result expected when cells from " controls " are transplanted into these birds, so that an alteration of the tumour to a type which will grow in the " non-susceptible " birds cannot be the cause of the sudden growth. Study of the testes of birds killed a few days after injecting acenaphthene showed that the process of cell division had been interfered with by this substance.

Experiments with 2 : 4-*dinitrophenol.*

It has been shown that 2 : 4-dinitrophenol and related compounds will cause a marked increase in the rate of carbohydrate metabolism of both normal and neoplastic tissues (Dodds and Greville, 1933, 1934) and a marked pyrexia is produced when these chemicals are injected into animals (Tainter and Cutting, 1933). Either effect may be expected to modify the rate of growth of a neoplasm. Vannfält (1936) reported that the substance had no effect upon tar-induced tumours in mice, and Emge, Wulff and Tainter (1933) stated that a transplantable rat tumour continued to grow after treatment, though some destructive changes were found in the body of the tumour. They considered that the surface of the tumour showed an increased growth.

A solution of 2 : 4-dinitrophenol was prepared by dissolving it in a little NaOH solution and neutralizing this with HCl, and then diluting to 1 per cent. strength. This solution was given intravenously. The maximum dose tolerated by the chicks was 0·5 to 1·0 c.c., depending upon the size of the birds. Four birds survived when such cases were given for eight consecutive days. There was no apparent effect upon the progress of the tumour, nor was there any change in the rate of growth after the treatment was discontinued.

Experiments with methylcholanthrene.

For an account of the many studies on the effect of chemical carcinogens on neoplasms, the comprehensive reviews of Cook and Kennaway (1938, 1940) and the papers of Haddow and his collaborators (Haddow, 1935, 1938 ; Haddow and Robinson, 1937, 1939 ; Badger *et al.*, 1942) should be consulted.

A 1 per cent. solution of methylcholanthrene in arachis oil was made in the same way as described for the acenaphthene solution, and 0·5 c.c. injected into the left leg of the following birds : (1) Ten birds of the Non-Susceptible strain, 28 days after the test inoculation of Rous agent ; the tumours of three birds had regressed, the others all bore small tumours. (2) Two Non-Susceptible birds whose tumours had regressed, treated 42 days after the test inoculation. (3) One "Control" bird with small tumours present 43 days after inoculation with Rous agent. (4) Two normal eight-week-old chicks. The latter showed no definite reaction to the inoculation over a period of eight weeks. (Neither has the arachis oil any effect upon Rous-infected birds ; see acenaphthene section.) Among the Rous-infected birds, in one case the tumour showed a marked increase in its growth-rate. This bird, which was one of those of Group (1), had a small tumour in the right breast. Within a few days of the injection of methylcholanthrene the tumour began to grow rapidly, and the bird was killed when moribund 20 days after the injection with a large tumour filling the whole of the right breast. In all other cases in which a tumour was present before inoculation these tumours began to decrease in size, and by the end of the third week they were no longer palpable, nor were any signs of them detected later post-mortem. All birds of the first three groups, including the animal whose tumour was stimulated, developed a very large swelling of the treated leg about 9 days after the injection of the methylcholanthrene. One bird was killed and examined at this time. The swelling involved the whole leg and extended into the lower part of the breasts. Between the skin and muscles was a layer of yellow gelatinous matter. When the leg-muscles were cut, a watery fluid containing numerous fat droplets oozed from them, and compression by forceps expelled a further large amount of similar fluid. A few blood-spots were seen in the muscles, but nothing else of an abnormal character. The swelling slowly subsided in the remaining birds and a few lumps began to appear in various parts of the leg, and by the end of the third week the oedema was no longer apparent. One bird killed on the 17th day after inoculation showed some oedema and a small tumour between the leg muscles. In another bird, killed on the 28th day after inoculation, no oedema was detected, but there was a large tumour in the leg. From about 28 days onwards the size of the tumours in the legs began to decrease, and by the end of 40 days, when the experiment was terminated and all birds killed and examined, only three of the birds had small tumours left. In two of these, tumours were also found in the lower femoral lymph plexus. No tumours were found at any other sites. Histological examination of all tumours found during this experiment was made. In each case the appearance was that of a typical Rous No. 1 sarcoma, though in two cases the number of mitotic figures seemed to be unusually high.

DISCUSSION.

This work was begun in order to determine whether the growth of the virus-induced tumours was more amenable to treatment by chemo-therapy than those in which an active virus cannot be demonstrated. By using the " Non-Susceptible " strain, a series of tumours not subject to marked variation in rate of growth was available, and at the same time the effect of diverse factors controlling the growth in the various birds was as far as possible eliminated. The substances used may have influenced the growth of the tumours in two ways, either by an effect upon the tumour, or by acting upon the mechanism responsible for the inhibition of growth of the tumour in the birds. The cause of this resistance is not yet known, but there is evidence that it is reduced when the growth-rate of the bird decreases, and is increased during laying, so that a failure to supply sufficient nutrition to the tumour may be tentatively assumed to be at least a part of the mechanism. The cause of the resistance sometimes shown by birds not of this strain has not yet been investigated. The occasional stimulation of the tumour sometimes shown during these tests may thus have been due to the action of the materials in checking the growth of the bird, and so rendering more nourishment available for the tumour. In the case of the colchicine tests, the increase in tumour size may also have been due to an increase in the amount of haemorrhage into the tumour, as this seemed to be more extensive in the colchicine-treated birds than in the others. Boyland and Boyland (1940) noted that a similar increase in the amount of haemorrhage was present in mouse tumours after treatment with colchicine.

The most interesting result is that obtained with methylcholanthrene. The regression of the tumours remote from the site of injection of the hydrocarbon is similar to the findings of Haddow and co-workers in their experiments on spontaneous and transplanted mammalian neoplasms. But at the same time it appears that the cells near to the site of injection of the methylcholanthrene are rendered abnormally sensitive to the action of the virus, and so a Rous tumour, apparently induced by the chemical carcinogen, develops at the point of injection. Since Mellanby (1938) has shown that the Rous virus will infect another non-filterable tumour growing in the same bird, the presence of Rous agent in these tumours was to be anticipated, and no attempt was made to pass them by way of cell-free preparations. It may be possible that a similar localization of a pre-existing virus was obtained when McIntosh (1933) obtained tumours which could be transmitted by a cell-free extract, after inoculation of chemical carcinogens into fowls. The failure of some others to confirm this finding could then be explained simply on the assumption that the particular birds used were free from such viruses. Even so, it is interesting to note that such tumours, though not containing an infective virus, have associated with them a " heavy protein " which chemically and physically resembles the Rous virus very closely. In these cases the localization of a " toothless virus " in the sense of Andrewes (1939) may not be an impossible conclusion. This interaction of the virus and chemical carcinogen recalls that found in the case of the tumours produced in rabbits treated with the Shope papilloma and afterwards with tar (Rous and Kidd, 1936). Further investigation of this interaction is likely to be of considerable interest in relation to the study of

carcinogenesis. For such work, of course, the "Non-Susceptible" strain is quite unsuited. Apart from this remarkable interaction it is worth noting that the Rous No. 1 tumour reacts to such diverse pharmacologically active substances as 2 : 4-dinitrophenol, colchicine and methylcholanthrene in the same way as most workers have reported for mammalian neoplasms.

SUMMARY.

No inhibition of the Rous No. 1 tumour was produced by 2 : 4-dinitrophenol.

Colchicine did not prevent the growth of established Rous tumours, nor would it inactivate the agent present in them either *in vivo* or *in vitro*. Normal birds treated with it showed typical development of tumours after infection with the agent. Treatment with acenaphthene produced irregular results, both regression and stimulation of the tumours resulting.

Injection of methylcholanthrene produced a violent local reaction in birds infected with Rous agent, with subsequent regression of established tumours and development of a Rous tumour at the site of injection of the hydrocarbon about three weeks later.

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THE ABSENCE OF A SEASONAL INFLUENCE UPON THE ROUS NO. 1 SARCOMA IN YOUNG CHICKS.

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THE fowl is unusual among laboratory animals in that many of its vital activities (ovulation, thyroid activity, moult, etc.) are controlled by seasonal influences. Consequently it may be suspected that a seasonal variation in cancer induction and growth would be exhibited in a more marked way than by the laboratory rodents usually employed for cancer research. There have been several reports that such an influence is evident.

Michalowsky (1928) stated that teratoma of the fowl's testis was readily induced by injection of zinc salts into the testis during spring, but not at other times of the year. The importance of the season in such experiments was also confirmed by Falin and Gromzewa (1939) and by Bagg (1936), who, however, was also able to induce teratomas at other seasons by simultaneous treatment with gonadotropic hormone. Peacock (1935) reported that the season had a marked influence upon the rate of growth and transmissibility of chemically-induced fowl sarcomas, the last six months of the year being the period of least activity of the tumours. The decrease in activity was so marked as to result in the loss of some strains of tumour. In a personal communication it was stated that this effect is still being shown. Peacock also noted that the loss of filterability of the Rous No. 1 sarcoma reported by Gye and Andrewes (1926) occurred during the season when chemically-induced tumours show least activity. Fränkel (1930) found transmission of Rous No. 1 by filtrates to be much easier during the egg-laying months of March, April, July and August. Recently Murphy and Sturm (1941) found that the interval between inoculation of a chemical carcinogen and the appearance of a tumour was greater during autumn than at other times of the year. On the other hand, many workers do not mention any seasonal variation, and ignore this factor in discussing their results. This suggests that in their experience it is not sufficiently important to influence their experiments.

In connection with investigations into the inheritance of the susceptibility of fowls to the Rous No. 1 agent, it became desirable to evaluate the possible effect of season upon the response of the 6-week-old chick to intramuscular inoculation with the free Rous agent. Records were available for the response of a large number of chicks of the Institute Brown Leghorn flock to inoculation with such material since November, 1935, and these were analysed to determine whether a seasonal variation was present. Only chicks aged 6–9 weeks

inoculated intramuscularly with a dose of agent of undoubted infectivity were considered. Many varied types of experiments were included, the use of cell-free Rous material being the only common factor. The early data are from the records of experiments performed by Dr. C. R. Amies, thus bringing the total number of birds considered to 971. The sexes were more or less equally represented in the experimental chickens.

The reactions to inoculation were grouped according to the following scheme : negative or regression ; small tumour ; large tumour. The size of the largest tumour 28 days after inoculation (or at death if caused by tumour before then) was taken to indicate the degree of susceptibility. Estimation of tumour size is to some extent subjective, but in the case of the Rous No. 1

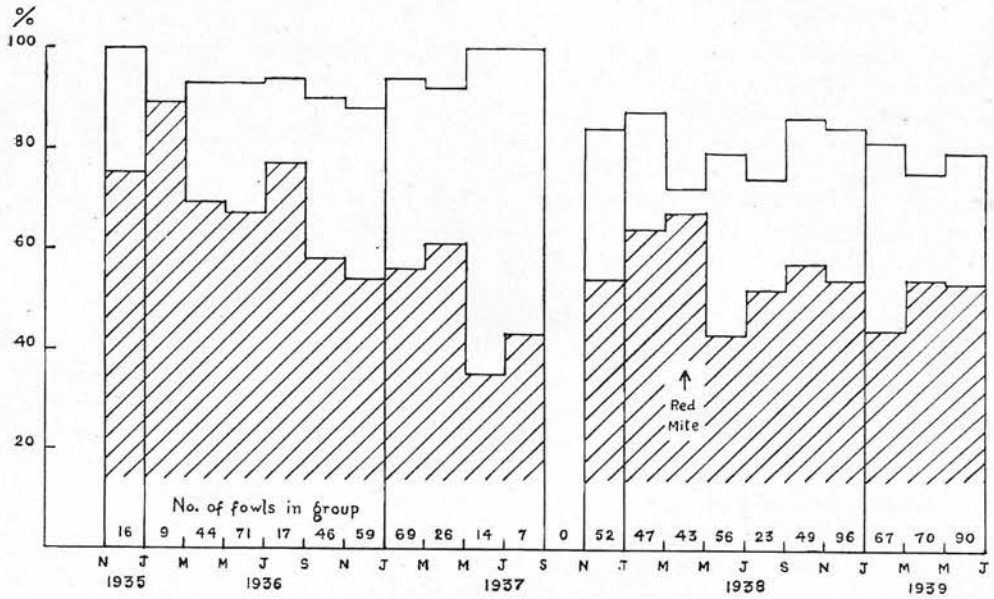

FIG. 1.—Showing the percentage of progressive tumours (white) and large tumours (shaded) induced each two-monthly period.

the two classes of size are readily separated in the vast majority of cases. The fowls were arranged in groups comprising birds inoculated during successive 2-monthly periods, which gave a reasonable number of animals in most groups.

A seasonal influence, if present, may affect either the establishment of a tumour, or its rate of growth. This would cause a variation in the number of progressive or large tumours respectively. The data are summarized in the figure. It will be seen that the numbers of progressive tumours remain fairly constant, and that the proportion of large tumours, though more variable, does not show a seasonal fluctuation.

Since 1941 work has continued upon the same stock of birds, and of the same age ; only males were used in this period. The number used was fewer, and consequently the results are of lesser significance, and are not given in

detail. Again there was no indication of a seasonal influence. In this connection it should also be noted that the line of birds bred at this Institute for resistance to inoculation with the Rous agent (as determined from an inoculation given at 6 weeks of age) does not show a loss in resistance at any period of the year.

It is interesting to note that there is a drop in both curves in the spring and summer of 1938. This coincided with a heavy infestation by red mite, apparently acquired during transit to the Lister Institute (the only occasion when any large outbreak of disease or parasitism was encountered). As the infestation was prevented from spreading to all the birds, the effect upon the curves is only slight, but it was noted at the time that the tumour was much less active in the infested stock. This was to be anticipated, as it is known that ill-health of the host may reduce cancer growth (Rohdenburgh, 1918; Ewing, 1940). An example is provided by comparing the result of an experiment comprising 18 birds, kept in the infested animal house, with the average results for a number of birds determined about this time.

Tumour Response.

	Regression and negative.	+	++	+++	++++
Infested (per cent.)	22·2	50	11·1	5·5	11·1
Normal (per cent.)	12	15	17	31	25

DISCUSSION.

It is of interest that the fowls used in this work were from the same stock as many of those used by Peacock, in whose experiments a seasonal factor is marked. But the conditions are so different in the two types of work (use of agent-induced and non-filterable tumours, age of bird, etc.) that they are not in any way comparable. Indeed, it has been observed (Carr, 1942) that in adults of the resistant strain, the resistance to the Rous agent is modified by such seasonal factors as moult and laying. The present findings should therefore not be extended to conditions other than those which apply to this work. The absence of a seasonal variation in the conditions of this study is not surprising. After 30 years of passage the Rous tumour is probably near maximum virulence, and the immature chick has not yet developed the endocrine mechanism which responds to seasonal influences. In addition, the first six weeks of the chick's life are spent in an environment artificially maintained at optimum conditions. A significant seasonal variation in such chicks does exist (Galpin, 1939), but is of less importance than individual variations.

The decrease in tumour growth associated with parasitism is of some interest. It is obvious that in a parasitized stock the degree of parasitism will vary according to seasonal changes or alterations in experimental conditions, and thus induce a variation in the tumour growth in the hosts. The importance of using disease-free stocks in such studies cannot be stressed too highly.

SUMMARY.

No definite seasonal variation in the susceptibility to the Rous No. 1 sarcoma has been found in healthy young Brown Leghorn chickens. It is noted that parasitic infestation may produce a seasonal alteration in susceptibility.

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SOME INVESTIGATIONS UPON THE NATURE OF THE RESISTANCE OF AN INBRED LINE OF FOWLS TO THE DEVELOPMENT OF THE ROUS No. 1 SARCOMA.

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UNDER a scheme sponsored by the Scientific Advisory Committee of the British Empire Cancer Campaign, the fowls of the pedigreed Brown Leghorn flock of the Institute of Animal Genetics were made available to workers using avian tumours. The susceptibility of the various individuals was noted and the results analysed. It became clear that parentage was an important factor in determining the degree of susceptibility of 6-week-old chicks to inoculation with the Rous No. 1 sarcoma agent, and it was found possible by selection to produce a line of birds, known as the Non-Susceptible or N-S line, which was very resistant to inoculation with the Rous No. 1 agent. The genetical aspects of this work will be dealt with in separate publications by Dr. Greenwood. The present account deals with some preliminary investigations into the nature of this resistance.

It should be emphasized that the N-S line was established and tested before the breeding stock had ever been in contact with birds bearing experimental tumours. Furthermore, spontaneous neoplastic diseases are extremely rare in the Institute flock. Acquired immunity resulting from infection with experimental material or spontaneously developed tumours (either of the animal or its parents) could thus be ruled out as complicating factors.

Response of N-S Birds to Inoculation with Tumour Agent.

All N-S birds are tested for susceptibility to Rous No. 1 agent at the age of 6 weeks (see Carr, 1942*a*, for details). In Table I is shown as an example of the difference between the response of this line and " controls " (i.e. birds taken at random from other Institute lines) data obtained over a period of 1941. The difference between the two is slightly reduced, because it was found necessary to kill about 11 per cent. of the control birds before maximal tumour development in order to provide material for experiments. Seasonal variation in response is shown neither by the N-S nor by the " control " birds (Carr, 1942*c*). The sexes contribute approximately equally to each group.

TABLE I.—*Showing Degree of Response of 6-week-old N-S and Control Birds to Inoculation with Rous No. 1 Agent.*

	Number tested.	Tumour response as percentage				Regression.	Negative.
		++++.	+++.	++.	+.		
N-S line .	128	0	0	1	28	63	8
Controls .	118	18	25	33	16	7	0

As a general rule the growth of the tumours in both the N-S and control fowls is similar for about the first 14 days. The N-S tumours then appear to lag behind the controls ; many become distinctly harder than the controls and remain about + in size, while the rest suddenly soften and completely or almost completely disappear by about the 22nd day. In the case of these regressions, a small tumour sometimes persists at the surface of the muscle or in the skin ; these are not classified as regressions. In the majority of birds regression appears complete, as far as can be judged macroscopically at post-mortem examination. Those tumours which persist in the N-S birds show a very slow and steady growth or regression. The results are independent of the amount of agent given, over the ranges used (10–1,000 minimal infective doses).

Effect of Age on Resistance.

In a previous paper (Carr, 1942*a*) a fall in the resistance of older N-S birds was described, which in several instances led to a redevelopment of Rous tumours without further inoculation. A series of susceptibility tests were carried out upon a number of day-old chicks, employing the same technique as for the 6-week-old fowls. As Table II shows, the tumour grew more vigorously in both N-S and control day-olds than in the older birds, but the N-S birds were still obviously less susceptible than the controls.

TABLE II.—*Showing Response of N-S and Control Birds Inoculated with Rous No.* 1 *Agent as Day-olds.*

	Tumour response.				Regression.	Negative.
	++++.	+++.	++.	+.		
N-S line .	0	7	8	15	11	3
Controls .	6	10	1	0	0	0

Metastases were frequently found in both groups, whereas they have not been noted in N-S fowls inoculated at the age of 6 weeks. It was observed that tumours sometimes appeared in the day-old birds at inoculation sites which parallel tests on 6-week-old animals showed to be quite devoid of active agent. As a control, some day-olds were injected with Rous agent into the breast and saline into the legs. A few tumours appeared at the site of the saline injections, indicating that the anomalous tumours were secondaries forming at points of injury. It should be noted that, as a consequence of this, comparative tests of two or more agent preparations in one animal are unreliable if day-old chicks are used.

Relation of Antibodies to Resistance.

At the beginning of this work the possibility that serum antibodies or other serum neutralizing factors might be the cause of this resistance was envisaged. Accordingly, experiments were early undertaken (while working at the Lister Institute, London) to investigate this point, before any of the tested fowls were returned to Edinburgh for breeding purposes, thus avoiding any complications due to immunity resulting from transmission of tumour agent to the experimental birds. The details of one such experiment will be described, and the others, which gave the same result, summarized.

Experiment 1.

Blood was taken from the wing vein of N-S fowls L.355 and L.367, and from control fowls L.397 and L.411, immediately on receipt from Edinburgh. All were 6 weeks old. The sera obtained were used in the neutralization test.

A cell-free Rous No. 1 agent preparation was inoculated into these fowls in the usual manner employed for testing susceptibility, and the same preparation was used for testing the neutralizing power of the sera.

Susceptibility results :

L. 397. Tumour size ++++, 30 days after inoculation with agent.
L. 411. ,, ,, ++++, ,, ,, ,,
L. 355. ,, ,, +, ,, ,, ,,
L. 367. No tumours resulted.

The suspension of agent used produced tumours in all of three susceptible birds when diluted 1/10, and in 1 out of 3 when diluted 1/100 ; 0·5 c.c. was inoculated.

Serum test : Mixtures of 2 vols. of undiluted Rous agent suspension + 1 vol. serum were incubated for 1 hour, and 0·5 c.c. inoculated into 6-week-old " controls " as shown in Table III.

TABLE III.—*Showing Absence of Neutralizing Antibodies in N-S Sera.*

The size of the tumours is indicated by the number of + signs.

Inoculum.			Site.		Fowl No. 942.	943.	949.
Agent suspension	+	serum	L.397	Right breast	+++	*	+
,,	,,	,,	L.355	Left ,,	+++	*	+
,,	,,	,,	L.397	Right leg	++++	*	+
,,	,,	,,	L.367	Left ,,	++++	*	+
					945.	946.	947.
,,	,,	,,	L.411	Right breast	+++	+++	+
,,	,,	,,	L.355	Left ,,	+++	+++	+
,,	,,	,,	L.411	Right leg	++++	+++	+
,,	,,	,,	L.367	Left ,,	++++	+++	+

* Indicates tumours regressed.

It is obvious that there were no detectable neutralizing antibodies in the N-S sera. Similar experiments on other fowls, carried out in essentially the same way as Expt. 1, always produced the same result. These are summarized below.

Expt. 2. Fowl L.298, susceptibility +, compared with L.497, susceptibility ++++.

Expt. 3. Fowls L.1843 and L.1938, susceptibility 0, each compared with L.1764, L.1916 and L.1933, susceptibility of each ++++.

Expt. 4. Fowls L.2394 and L.2398, susceptibility +, each compared with L.2143 and L.227, susceptibility of each ++++.

Expt. 5. Fowl L.2356, susceptibility +, compared with L.2397, susceptibility ++++.

All these experiments failed to give any indication that N-S sera contained any neutralizing substance not present in the sera of susceptible birds. A further test was carried out after the introduction of some tested fowls into the N-S breeding stock, the serum on this occasion being taken from the chicks 7 days after the testing dose of Rous agent was inoculated. Once again the N-S sera failed to inhibit the activity

of a Rous agent suspension. It thus seems clear that preformed antibodies cannot be the cause of the resistance of these birds.

Although antibodies to the Rous No. 1 agent are often present in " normal " fowls, they are usually found only in older animals (Andrewes, 1931 ; Ledingham and Gye, 1935 ; Amies, 1937 ; Duran-Reynals, 1940). Amies found no indication of their presence in an extensive series of young chicks. Such antibodies would rather be expected to result in failure to produce a tumour after inoculation of agent, whereas Table I indicates that the N-S line characteristically produces regressing tumours. Furthermore, the breeding experiments have indicated that the sire is an important factor in determining the degree of resistance of the chicks. This would not be expected if the resistance were due to a neutralizing substance transmitted from the dam *via* the egg. These facts provide additional evidence that antibodies are not the cause of the resistance exhibited by the N-S line.

Transplantation of Rous Cells into N-S Birds.

If the failure of the Rous tumour to develop in the N-S birds were due to some factor in the cells which was inimical to their cancerous development, then transplants of tumour cells from a progressive tumour should result in proliferation of the graft. Implants of cells from routine Rous No. 1 tumours into 14 N-S birds whose agent-induced tumours had regressed showed undoubted growth in only one case. It might be objected that these grafts were eliminated by iso-antibody action. Opportunity was therefore taken of the finding that progressive tumours may sometimes appear in N-S fowls after treatment with acenaphthene or methylcholanthrene to transplant progressive N-S tumours, but without any greater success (Carr, 1942*b*). The cause of the resistance is thus not to be found in the induced tumour cells. The reverse experiment of grafting N-S tumours into control fowls was not considered practicable, owing to the possibility of infecting the host cells with agent liberated from damaged tumour cells.

Growth of Other Tumours in N-S Fowls.

By the courtesy of Dr. Peacock, a specimen of the chemically-induced GRCH/15 sarcoma was obtained, and has been maintained at this Institute. All control fowls inoculated with cells of this tumour have grown progressive tumours. Grafts into 25 N-S birds whose agent-induced tumours had regressed resulted in only 14 birds yielding progressive tumours, and grafts into 2 untested N-S birds grew slightly and then regressed. The tumours which did grow all resembled typical GRCH/15 sarcomas in rate of growth, macroscopic and microscopic appearance. The N-S line thus shows a distinct resistance to the growth of this non-filterable sarcoma as well. Unfortunately the fowls thus tested were offspring from 11 dams, so that the data is insufficient to show whether these differences in response were due to inherited factors. But it was noted that of the two dams who had 4 offspring tested, L.352's offspring all showed tumour growth, while those of L.1848 failed to do so. L.352 was related to the N-S line, but had not been tested herself. Her offspring were all as resistant to Rous agent as N-S fowls.

In order to avoid the risks of cross-infection no other avian tumours are being maintained, but a consideration of susceptibility results obtained in earlier work indicated that a high proportion of fowls resistant to the Fujinami sarcoma and the Des Ligneris sarcoma (which is very similar to the Fujinami (Amies, Carr and Purdy, 1939)) were related to the N-S line.

DISCUSSION.

In spite of a large amount of experimental work, little is really known about the subject of resistance to neoplastic growth. The resistance often exhibited to transplanted tumours is now considered to be due chiefly to iso-antibody action, and hence can offer no possibility of leading to any measures of therapeutic value for the treatment of spontaneous neoplasms. The study of the susceptibility of mice to spontaneous mammary neoplasms has shown that this is an exceedingly complex phenomenon involving hormones, milk-transmitted factors, maternal influences and chromosomal factors, whose disentanglement is only beginning. Investigations upon resistance to the action of chemical carcinogens have scarcely started. The N-S line of fowls thus represents a very valuable additional line of attack upon the problem of resistance and susceptibility to cancer.

The present work is only a preliminary orientating investigation into the nature of this resistance, about which little can yet be said. But the fact that the resistance is so obviously directed against *developing* tumours makes it of extreme interest from the point of view of possible therapeutic applications, which is enhanced by the suggestion that it is not confined to a single type of tumour.

As the N-S line was established solely on the basis of experimentally determined resistance to the Rous No. 1 agent alone, it is difficult to believe that an independent resistance to Fujinami and GRCH/15 tumours was unwittingly fixed simultaneously. It seems much more probable that a general resistance to tumour growth is involved. A fuller discussion of the data bearing upon this point will be given in the descriptions of the breeding experiments. Such a general inherited resistance to neoplasms should be of great interest to the poultry industry, in which losses due to such diseases are notoriously high.

It is interesting that the birds show their greatest resistance to Rous tumour agent at the age of about 6–10 weeks, at which time the animal itself is growing most rapidly. The term " resistance " is, of course, a compound of many factors. For instance, a tumour reaching a weight of 10 g. is a burden to a 3-week-old chick, but of little consequence to an adult ; differing rates of vital processes between young and old fowls may result in varying rates of growth of established tumours, and varying reactions to the toxic effects of necrotic portions of tumour. All these will influence the degree of resistance of the bird, and should be separately evaluated in a discussion of the variation of resistance with age. A simple variation need not be expected, and the data presented is not suitable for an elaborate consideration of this aspect of the problem.

The type of variation noted is, however, not consistent with the suggestion that the resistance of the N-S chicks is due to any protective substance transmitted from the dam *via* the egg, in which case the youngest chicks would be expected to have the greatest resistance. It was fortunate that the separation of the breeding and experimental work in the early days permitted a test at this point without any of the possible complications arising. All the evidence available suggests that such protective substances play no part in the resistance shown by the N-S birds. That resistance can exist independently of protective serum antibodies has been demonstrated for isolated cases by other workers (Andrewes, 1931 ; Duran-Reynals, 1940 ; Troisier, 1934). This, of course, does not mean that such antibodies may not in any circumstances contribute to host resistance. But the resistance of the young N-S fowls must be primarily due to other, as yet unknown, factors.

SUMMARY.

Inoculation of Rous No. 1 agent into 6-week-old fowls of the N-S line typically

produces small tumours, most of which regress between the 14th and 22nd day after inoculation. This resistance is present in day-old chicks, but to a lesser extent, and is decreased also in older birds. It is not due to serum antibodies. The resistance is exhibited also to implanted Rous cells, GRCH/15 cells, and possibly to other filterable tumours.

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THE RELATION BETWEEN AGE, STRUCTURE, AND AGENT CONTENT OF ROUS No. 1 SARCOMAS.

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It is well known that extracts, or other preparations of cell-free agent from the Rous No. 1 sarcoma, as well as other filterable fowl tumours, vary very much in the amount of active agent contained in them, even when the same technique is scrupulously followed in each experiment (Rous and Murphy, 1914 ; Gye and Andrewes, 1926 ; Baker and McIntosh, 1927 ; Cramer and Foulds, 1930 ; Gye and Purdy, 1930, 1931 ; Doerr, Bleyer and Schmidt, 1932 ; Amies and Carr, 1939 ; Carr, 1942). Not only may the agent be totally lacking, but the extract may even have an actively inimical action upon added agent—the "inhibitor action" (Gye and Purdy, 1931 ; Sittenfield, Johnson and Jobling, 1931 ; Murphy and Sturm, 1932). As the causes of these variations have not been understood, their occurrence has seemed capricious in nature. Thus Cramer and Foulds (1930) remark, "It is impossible to predict whether a particular Rous sarcoma No. 1 will yield a completely inactive filtrate."

In connection with studies upon the cause of the resistance of a line of fowls to the development of Rous No. 1 tumours, it became necessary to undertake a study of these phenomena. It was found that not only could this prediction be made, but that the activity of any agent preparation could be foretold with reasonable accuracy in most experiments.

Determinations of the amount of agent per gramme of tumour were available for 61 tumours, together with records of size, rate of growth and structure. The hosts were all Brown Leghorns of the Institute flock, aged about 6–9 weeks at the time of inoculation of the tumour material. Most of them were males, and the majority had been injected with cell-free material. Otherwise they had little in common. They were from various inbred lines and bore single or multiple tumours, due either to injection of plain tumour extract, or mixtures of agent with antiserum, inhibitor, or other materials. The activity was variously determined upon extract, or material recovered after various methods of processing. The time of extraction varied from 1 hour to over 30 hours. In all experiments hydrocyanic acid was present as an inhibitor of oxidation. Agent content was determined by injecting suitable decimal dilutions of the final product into two or three susceptible chicks. In spite of the increased chances of altered activity thus allowed by modifications of technique, the activity showed a

rather consistent variation with the age of the tumour alone. As shown in the figure, all tumours processed up to the 13th day after inoculation of the host contained 10^6 or more agent particles per gramme of tumour. Between the 14th and 22nd days the activity was often less than this, but never below 10^5. Later than this, the activity was less than this in about half the material taken. No active agent was found after the 40th day, while extracts of all tumours taken before this time contained some agent. Considering the heterogeneous nature of the material, and the crude approximation to the agent content obtained by titrating decimal dilutions, the scattering of the points is not very extreme, and it is seen that a fairly reliable indication of the extracted agent can be obtained from a consideration of the age of the tumour.

On the other hand, the structure of the tumour, which has often been considered to give an indication of the amount of agent obtainable in extracts (Gye and Andrewes, 1926 ; Gye and Purdy, 1930, 1931), was, in this series, found to be of very slight value.

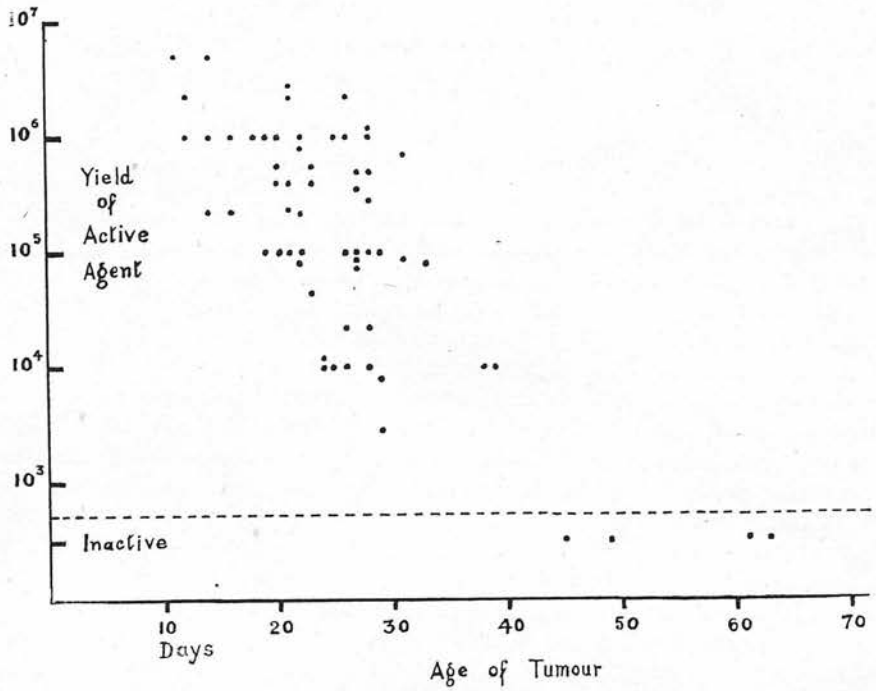

FIG. 1.—The amount of agent extracted per gramme of tumour from tumours of varying ages.

It was noted, for example, that three of the tumours of 16–22 days' growth were slowly growing, and white or yellow and rather hard when taken, yet their agent content was as high as that of rapidly growing tumours of the same age. This failure of structure and appearance to give any reliable clue to the content of agent extractable from the tumour was noted throughout the course of this work. As an illustration of the type of finding in the case of tumours of recent growth, the following experiments are given in detail :

Experiment 1.—Fowls 652 and 653 were inoculated in both breasts and both legs with a Rous extract.

Fowl 652 proved extremely susceptible to the Rous tumour agent, and was bearing large tumours when killed on the 16th day after inoculation. These were soft, pink, fungating, very malignant growths, in which a good deal of haemorrhage had occurred

—in fact, they were typical examples of this tumour in its most virulent form. The tumour in the right breast was processed by the method of Amies and Carr (1939), and yielded 10^5 agent particles per gramme of tissue.

Fowl 653 was much more resistent to tumour growth. At the end of 19 days after inoculation the tumours were only about one-third of the size of those of 652 at death. They were found to be compact, very yellow, quite firm, and were not invading the surrounding muscles in the marked fashion noted in Fowl 652. Only one tumour showed any signs of haemorrhage, and this was in a tumour in the leg, and was believed to be due to rupture of a small blood vessel caused by muscular action immediately after death. The tumour corresponding to that used in Fowl 652 was processed by the same method, and the same yield of tumour agent obtained.

Experiment 2.—One of the animals used to titrate the agent of Fowl 652 grew rather small and slow tumours. The bird was killed on the 14th day after inoculation, and the largest tumour taken. It was pale yellow, firm, and with little invasive tendency. The centre was more yellow and was discarded as being probably necrotic. The remainder of the tumour was processed by the same method as used in the previous experiment and yielded 5×10^6 agent particles per gramme of tissue.

Experiment 3.—Fowl 530 was inoculated with varying dilutions of a routine agent preparation. Tumours appeared in all sites, and grew quite rapidly. The largest was noted to be unusually soft, and when the bird was killed on the 16th day after inoculation it was found to consist of soft fragments of tumour tissue growing from the edge of the tumour or lying loose in a semi-fluid matrix. Portions of the tumour tissue were removed and processed by the same method as before, and the product yielded 5×10^6 agent particles per gramme of tissue.

Similarly, it was found that tumours which grew rapidly, and had the characteristic appearance of such tumours, did not necessarily contain a large amount of extractable agent. One such case will be described in detail :

Experiment 4.—The agent-induced tumours of Fowl 640 grew very slowly at first (a companion bird inoculated with the same material was killed with very large tumours after 22 days). About 40 days after inoculation the tumours began to increase in size very rapidly, and the animal was killed on the 49th day bearing very large tumours, all soft, fungating, haemorrhagic, very malignant, and typical of the rapidly-growing type of Rous No. 1 tumours. A plain 10 per cent. cell-free extract of this material was found to be devoid of any agent activity.

Two of the other tumours of more than 40 days' growth were also the result of a similar spurt of growth, and had a similar structure, yet no active agent could be extracted from them. Appearance seems to be of value only in indicating that a large tumour of the slowly-growing type must be an old one, and hence only a small yield of extractable agent is to be anticipated.

It was also noted that the results gave no support to the claim (Cramer and Foulds, 1930) that slowly-growing tumours result from " attenuated agent " obtained from tumours of slow growth and low agent content. The rate of tumour growth appeared to be determined by the susceptibility of the host (as seen, for example, in Experiments 1 and 4), and neither the source nor the amount of agent seemed to have any influence. It has been shown that this varied susceptibility of the Institute flock is often due in part to inherited factors (Greenwood, 1940 ; Carr, 1943 ; and unpublished findings). Many other experiments showing that the rate of growth of the tumour is dependent upon the susceptibility of the host and not on the source of the tumour agent are to be found in these papers.

This concept of variation with the age of the tumour will also account for the non-filterability of the recurring tumours previously described (Carr, 1942). Although

several of these were rapidly-growing sarcomas, they developed several months after the initial stimulus of tumour material was first experienced by the fowl, and some tumour material must have been present, though latent, until the recurrence of the tumours. These tumours should therefore be considered as equivalent to tumours of several months' duration, and the absence of any agent in the extracts is in agreement with the suggestion that activity and age are inversely related.

So far only animals inoculated at the age of 6–9 weeks have been considered. It requires only a slight extension of the concept described above to deal with the case of animals inoculated at a more advanced age. While the serum of young chicks has no anti-Rous activity (Amies, 1937), older birds often contain " naturally-occurring " antibodies to the Rous No. 1 agent. Whether this is due to agent remaining in the bird without causing tumours, as occurred in the experiments of Rous, Murphy, and Tytler (1912), Pentimalli (1924), and in the case of the recurring tumours just mentioned, or to a related virus with a common antigen, or to a heterologous antigen, is a matter for further investigation. But it is reasonable to ante-date the " tumour stimulation " in these cases to a period before the inoculation of the experimental material, and thus to expect that such fowls would be especially prone to bear tumours yielding inactive extracts. This is well known to be the case. Furthermore, as commercial hatching is usually carried out almost exclusively in the spring, it might be expected that workers who use animals from such sources would find that the activity of tumour extracts would fall progressively throughout the year, but increase again in spring. The variations reported by Gye and Andrewes (1926) and by Fränkel (1930) are significant in this connection.

DISCUSSION.

In this work only the Rous No. 1 sarcoma has been considered. It is seen that the age of the tumour is the most important factor in determining the activity of the extract prepared from it. Many other factors will, of course, modify this relationship. In particular, very young chicks seem to be abnormally responsive to Rous agent (" haemorrhagic disease " of Duran-Reynals, 1940), and the effect of age may be less in such animals. A limited number of experiments on such animals have suggested that this is so, but the data are insufficient for a definite conclusion to be drawn.

The decrease in activity is probably due to an inhibitory substance elaborated by the fowl or the tumour, as in certain types of experiment the agent can be separated from such a material, e.g. by high-speed centrifugation or absorption (Sittenfield, Johnson and Jobling, 1931 ; Murphy and Sturm, 1932 ; Claude, 1939). Use of such techniques should confuse the relationship demonstrated above. Though in this work no fowls of the N-S strain bred for decreased response to Rous agent are included, it was found that data from them fitted into the scheme quite readily, and that activities were easily predicted by considering the age of the tumour alone.

It is the experience of workers using other filterable fowl tumours that filterability varies in a fashion similar to that found for the Rous No. 1 tumour, with some modifications due to the special characteristics of the tumour strain employed. A complete discussion of this, and of the mechanisms involved, is deferred until the experiments on the " inhibitor " are described. But it seems desirable to point out that this variation with age due to an inhibitor seems to be common to all tumours induced by a virus-like intracellular agent. Therefore experiments designed to prove the existence of such agents are more likely to succeed if very young grafts are used. The increase in tumour material obtained with older grafts may not compensate for the reduction

in the amount of tumour agent which may result. It also follows that a slowly-growing tumour, though bearing a filterable agent, will be non-filterable for the greater part of its life in any host.

SUMMARY.

The amount of active agent extracted from Rous No. 1 tumours was inversely proportional to the duration of growth in the host. After 40 days all tumours were non-filterable ; before then all contained some agent. The appearance of the tumour bore no relation to the amount of agent obtained in extracts.

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PROLONGED ANTIBODY PRODUCTION FOLLOWING RECOVERY OF FOWLS FROM ROUS No. 1 SARCOMA.

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As is well known, there are two types of immunity following virus infection ; after certain diseases there is a transient period of immunity, while others confer a very prolonged, sometimes life-long immunity. It is usually considered that the latter type of immunity results from the virus remaining latent in the host, corresponding somewhat to the " carrier " stage of bacterial infections, though rigorous proof of the presence of the virus is not always available. It has been recognized from the beginning of work on the filterable fowl tumours that certain fowls may be resistant to the inoculated agent, and that if they recover from the infection they are immune to further inoculation (Rous and Murphy, 1914). The exact duration of this immunity has not been studied, and it remains a matter of controversy how far this immunity is due to humoral antibody, and how far it is due to other types of resistance. Though such fowls have often been used for immunological studies on the fowl tumour viruses, workers seem to have had little faith in the ability of the fowl to maintain a high antibody titre, and have usually preceded their investigations by a series of immunizing injections of tumour material.

In the course of work which involved keeping fowls for a considerable length of time after they had recovered from a single injection of Rous No. 1 virus, it was found that tumours would sometimes recur in these birds many months after the first induced tumours (if any) had vanished, and Rous No. 1 virus was recovered from grafts of these tumours (Carr, 1942). It thus became necessary to postulate the existence of the Rous virus in a latent form in these birds, and consequently it might be anticipated that such birds might have constant high antiviral antibody titre in their serum. Investigations were therefore carried out to see if this was the case, and to obtain information as to how often this would be found, thus obtaining information as to what proportion of birds have the virus remaining latent in the tissues after recovery from the tumours.

It was found without exception that sera obtained from birds which had recovered from induced Rous No. 1 tumours some time previously possessed strong virus-neutralizing activity when tested *in vitro*, irrespective of the time which had elapsed between taking the blood sample and the inoculation with tumour material. Of many tests performed, the following four are selected as examples because they belonged to a small group of fowls kept in individual cages and under constant observation. All were females which had been tested for susceptibility to Rous agent at the age of six weeks by the method previously described (Carr, 1942).

Experiment 1.—Fowl 0.135. For previous history, which included a temporary recurrence of the Rous tumour, see Carr (1942).

Fowl 0.1262. Tested 13.viii.41, the largest dose of agent containing over 1000

minimal infective doses (M.I.Ds.). Small tumours appeared in the breasts and soon regressed.

Blood was withdrawn from these two fowls and from a young normal chick on 6.viii.42, and the sera removed from the clot next day. The serum, or a 1/10 dilution of it, was mixed with an equal volume of a cell-free Rous agent suspension diluted to such an extent that 0·5 ml. of the mixture contained about 50 M.I.Ds. of agent. The mixture was incubated for two hours at 37° and then left in the cold room overnight. Next day the activity of agent in the various mixtures was determined by inoculating 0·5 ml. into the breast or leg muscles of susceptible chicks as shown in the table.

Table Showing Neutralizing Action of the Sera taken from Birds which had Recovered from Rous Tumours.

Inoculum.	Fowl 525.	Fowl 526.
Rous agent + normal fowl serum	+++	+++
,, ,, + 0.135 ,, ,, ...	—	—
,, ,, + normal ,, ,, × 1/10 . . .	+++	+++
,, ,, + 0.135 ,, ,, × 1/10 . . .	+	++
	Fowl 527.	Fowl 528.
,, ,, + normal ,, ,, ...	+++	++
,, ,, + 0.1262 ,, ,, ...	—	—
,, ,, + normal ,, ,, × 1/10 . . .	+++	++
,, ,, + 0.1262 ,, ,, × 1/10 . . .	—	+

The size of the tumours when the birds were killed is indicated by the number of + signs.

Experiment 2.—Fowl 0.625. Tested 30.v.41, the largest dose containing about 1000 M.I.Ds. A small tumour appeared at the site of the largest dose, but soon regressed. The subsequent history of the bird was in no way remarkable. No recurrence of the tumours was noted. A blood sample was withdrawn 27.x.42, and tested by the same technique as in Experiment 1. Serum diluted to 1/4 and 1/40 completely inactivated 100 M.I.Ds. of agent contained in an equal volume of suspension.

Experiment 3.—Fowl 0.619. Tested at the same time, and in the same way, as 0.625. Tumours appeared in both breasts, and lasted rather longer than in 0.625 before regressing. A blood sample was withdrawn 13.iv.43, and 3 ml. of the serum obtained completely neutralized the activity of 30 ml. of agent suspension containing over 10,000 M.I.Ds. As confirmatory evidence that this inactivation was due to neutralization by antibody and not to another type of virus destruction, the mixture of virus and serum was centrifuged and active virus recovered from the deposit. That this separation of agent and antibody is also found with serum obtained from immunized fowls was previously shown by Amies and Carr, 1939.

DISCUSSION.

The neutralizing power of these sera was quite as strong as that of sera taken from birds which had been submitted to a preliminary course of immunization. Exactly how the virus, presumably having an intracellular existence, can induce the formation of antibodies in serum which does not penetrate the cell-wall is not a problem peculiar to the virus of the Rous No. 1 sarcoma. There is no evidence that a diffusible specific soluble substance is produced as is the case with vaccinia ; but a similar problem also

exists in the case of many other virus infections, and is more likely to be solved by workers who use material more easily handled than are the fowl sarcoma agents.

The demonstration of active antibody in these fowls can, by analogy with other virus diseases, be regarded as proof of the presence of tumour virus, though tumours are not produced. As virus has been recovered from birds over a year after inoculation (Carr, 1942), it was known that it could remain latent in some birds. In the present investigation high antibody titres were found in all tested birds, so it would appear that this idea of latent virus must be extended to all birds recovering from Rous No. 1 tumours. The manner in which the virus can exist in the bird without producing tumours is unknown; but it is known to remain in the tissues in a similar inactive way after intravenous inoculation (Rous, Murphy and Tytler, 1912; Pentimalli, 1924) and when disseminated from growing tumours (Mellanby, 1938). The analogy of this condition to the "milk factor" of mouse mammary tumours is apparent.

The importance of this latent fowl tumour virus cannot yet be evaluated. Though birds infected with virus-induced tumours will not produce immediate epizootic outbreaks of neoplasms in a flock (Rous, Murphy and Tytler, 1912), there is no evidence that the virus is not transmitted in this latent form, either between fowls or *via* the egg. Should this occur, instead of an immediate outbreak of tumours, it would seem more reasonable to expect the disease to appear in a similar fashion to the manner in which it appeared in the case of the recurring tumours previously described, *i.e.* isolated occurrences of the tumour at irregular intervals in a proportion of the birds. The high incidence of neoplastic diseases in most flocks suggests that such an aetiology is not altogether improbable, and it is very unfortunate that there is no reliable evidence as to the identity or otherwise of viruses responsible for neoplasms in isolated poultry flocks.

SUMMARY.

Fowls tested one to two years after recovery from Rous No. 1 tumours all possessed a high content of neutralizing antibodies to the Rous No. 1 agent in their serum. This is regarded as evidence that the virus has remained in a latent form in the tissues of these birds.

All expenses in connection with this work were borne by the British Empire Cancer Campaign.

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EXPERIMENTS ON THE INHIBITOR OCCURRING IN ROUS No. 1 SARCOMAS.

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THE relation of the filterable tumours of fowls to other neoplasms has remained an outstanding problem of cancer research, and a satisfactory solution seems no nearer than when the first of these tumours was described by Rous in 1911. In general, the filterable tumours have been spontaneous growths, while fowl tumours induced by chemical agencies have proved non-filterable, though exceptions are well known (it is convenient to retain the conventional terms " filterable " and " non-filterable " to designate agent- or virus-transmitted tumours and those transmissible only by intact cells respectively) ; this work has been reviewed by Murphy and Sturm (1941). It has often been suggested, more or less explicitly, that the difference between the two types is only one of degree and not of kind, and that the non-filterable tumours are only cases in which the isolation of a filterable agent is for some reason or other of extreme technical difficulty (Gye and Purdy, 1930*a* ; Cramer and Foulds, 1930). This suggestion receives support from the many reports of the Rous No. 1 tumour itself being temporarily " non-filterable," the best example being that reported by Gye and Andrewes (1926). It is notable that many of these reports date back to the early days of work upon these tumours, before inactivation of the agent by oxygen was recognized, and the difficult feat of filtering a very viscous extract through highly-absorbing material was attempted, the filtrate being tested by inoculation into birds of dubious age and antecedents. After the demonstration by Ledingham and Gye (1935) that the agent could be separated from the extract and purified to some extent by a process of fractional centrifugation, and the subsequent widespread use of this method in place of filtration, together with the use of anti-oxidants during the processing as first recommended by Gye and Purdy (1930*b*), reports of inactive preparations seem to have become less frequent.

Many workers have demonstrated that some extracts of non-filterable agent-induced tumours will inactivate cell-free Rous agent *in vitro*. Experiments on this " inhibitor " action have been reported by Gye and Purdy (1931), Sittenfield, Johnson and Jobling (1931), Murphy and Sturm (1932*a* ; 1932*b*), Fraenkel (1938), and Claude (1939). Many other workers have reported experiments which appear to indicate the existence of this substance, and have stated that further work on the problem will be undertaken, though additional data have not yet materialized. This is probably in part due to the rarity and inconstant appearance of the phenomenon.

During the course of work on the inheritance of resistance to the Rous No. 1 sarcoma in fowls, it was found that a large proportion of the fowls kept for some time after the regression of any tumours produced by the testing inoculation suffered a recurrence of the malignant growth, and that all these tumours were examples of non-filterable

Rous tumours (Carr, 1942). It was also found that slow tumours induced in a resistant line of fowls and in certain other resistant birds were similarly prone to yield non-infective filtrates (Carr, 1943*b*). As described below, all these tumours that were tested showed the " inhibitor " effect. It therefore became necessary to investigate the nature and action of this " inhibitor " to determine what relation it bore to the resistance to the Rous tumour agent exhibited by these birds.

Occurrence of Inhibitor.

First of all, an analysis was made of the infectivity of extracts of agent preparations obtained from a series of Rous tumours of assorted types, from which it was concluded that the infectivity of the tumours varied chiefly with the duration of growth of the tumour in the host ; after 40 days' growth active agent could no longer be extracted from the tumour. This aspect of the work has been described in a previous paper (Carr, 1943*b*). This preliminary work much assisted further investigations by indicating which tumours could be expected to contain no extractable agent, and hence a probable high content of inhibitor, without previous testing being necessary. Of nine such tumours that were proved to be non-filterable, all were found to possess a marked inhibitor action.

As an example the experiment carried out on the recurring tumour of fowl O.274 will be described. The history of this bird has already been given (Carr, 1942). The tumour was preserved in glycerol, and the effect of this method of preservation was controlled by a parallel test on a specimen of the non-filterable fowl sarcoma GRCH/15 similarly preserved ; it was known that this tumour when fresh yielded an extract devoid of any marked anti-Rous activity, and did not induce the formation of anti-bodies to Rous No. 1 virus (unpublished).

Experiment carried out 5.xi.42.

1 g. of O.274 tumour taken on 16.xii.41 and 1 g. of GRCH/15 tumour taken on 16.vi.41, each preserved in 50 per cent. glycerol, were extracted with 5 ml. of Ringer solution, and the extracts clarified by centrifuging. The extracts and a fresh specimen of serum from a young chick were mixed with a suspension of Rous agent, incubated for two hours, and then injected as shown in Table I. Each inoculum was 0·5 ml., and contained about 20 minimal infective doses (M.I.Ds.) of agent when mixed.

TABLE I.—*Showing Neutralization of Rous Agent by Extract of a Non-filterable Rous No. 1 Tumour.*

Inoculum.		Site.	Fowl No. 618.	Fowl No. 619.	Fowl No. 620.
Rous agent	+ recurring Rous extract	Left breast	—	—	—
,,	+ normal fowl serum	Right ,,	+	+	+++
,,	+ GRCH/15 extract	Left leg	+	++	+++
,,	+ normal fowl serum	Right ,,	+	++	+++

The size of the tumours is indicated by the number of + signs.

Not only recurring tumours, but also those which were non-filterable by virtue of their slow and lasting growth, were similarly shown to contain a quantity of inhibitor.

Serum Antibody in Fowls Bearing Non-filterable Rous Tumours.

It was previously shown (Carr, 1943*c*) that all fowls recovering from Rous No. 1 tumours maintained a high content of agent-neutralizing antibodies in their serum.

As it was now possible to predict which tumours were going to be non-filterable, it became feasible to determine the antibody content of the serum of birds bearing such tumours. In all, seven birds were tested, and all were found to have a high content of neutralizing antibodies in their serum. As an example, an experiment carried out on a non-filterable recurring tumour will be given in detail.

Fowl O.646 was tested for susceptibility to Rous agent on 30.v.41 by the routine method (Carr, 1942), the largest inoculum containing approximately 100 M.I.Ds. of agent. Small tumours appeared in the breasts but soon regressed. No recurrence was noted up to 27.vi.42, but a small tumour was noted in the right breast 7.vii.42, which grew rapidly and killed the bird on 27.vii.42. A blood sample was taken on 9.vii.42, and 0·25 ml. of the serum or a 1 : 5 dilution of it completely inactivated 50 M.I.Ds. of agent contained in an equal volume of suspension. The recurring tumour yielded an inactive extract, though cell-grafts grew readily.

This once again illustrates the fact that serum antibodies cannot protect a fowl against the growth of tumour cells. But it is obvious that an extract of minced tumour from these birds will contain the agent suspended in a fluid which may be regarded as dilute antibody, and inactivation of agent is to be expected. Cell grafts, on the other hand, would be expected to grow normally, as antibody transferred by the graft would have no effect on the cells and would soon be diluted by the tissue fluids of the host and the inactivating action lost. It is thus apparent that some at any rate of the inhibiting action of the extracts that were investigated was due to antibodies. It remained to see whether this was the only inactivating material present. Though the suggestion that antibody and inhibitor are identical is not new, most workers have regarded this as improbable, chiefly because of the experiments of Murphy and Sturm (1932*b*), who claimed that "inhibitor" prevented the growth of certain mammalian tumours, while antibody had no such action. As the experimental evidence was that two out of four specimens of crude "inhibitor" reduced the growth of one out of three types of mouse tumours, while a smaller series of tests on an unstated number of antisera had no such action, this cannot be regarded as decisive proof that the two are distinct entities.

Fractionation of Extract Containing Inhibitor.

Claude (1939) reported experiments indicating that the inhibitor is protein in nature. In confirmation of this, it has been found in one experiment that the inhibiting material could be salted out by ammonium sulphate, and that this product could be dissolved in saline to give a solution with all the inactivating action of the original extract.

Flocculation of Tumour Virus.

Flocculation of fowl tumour agent when mixed with antiserum was first demonstrated by Ledingham and Gye (1935). It is therefore to be expected that if antibody and virus are together in the extract of a non-filterable Rous tumour some flocculation may be expected. This has been a constant feature of the present investigation of these tumours. It has been noted that cell-free extracts of tumours found to be non-filterable are much less stable than extracts of routine tumours, and that addition of extract of non-filterable Rous tumours to an otherwise stable active extract or purified agent suspension will cause flocculation. This indicates that the amount of antibody is relatively large. It is obvious that this formation of floccules will cause loss of agent in the preparation of cell-free extracts whether centrifugation or filtration is employed to prepare the extract, quite apart from the agent-inactivating power of the fluid. It was also noted that preparations of purified "virus" obtained from non-filterable

Rous tumours by carrying out the same procedure as will yield an active suspension of Rous agent from young Rous tumours are unstable in suspension. Though virus and " inhibitor," like virus and antibody, do not form an immediate stable and inactive complex, it is possible that some antibody will remain on the virus particles after separation has been attempted, thus causing a tendency to flocculate without causing complete inactivation. It was found that a stable active suspension of Rous virus became similarly unstable when recovered in an active form from an inactive mixture of virus and antibody by centrifuging. In passing, it might be noted that a similar action appeared to be a cause of complications in agglutination tests carried out on Rous virus suspensions. It was noted that suspensions made from old tumours were less stable and more sensitive to antibody than those made from young tumours by the same method. The difficulty of getting comparable results was so great that this line of research was abandoned.

The most complete examination of this flocculation was made on the tumour of O.646 referred to above, processed simultaneously with a routine 24-day-old Rous tumour ; these preparations, or the sera obtained from the corresponding birds, are referred to as N-F and F respectively in Table II. All agent suspensions and serum dilutions were made in saline containing 0·2 per cent. formalin, and equal volumes of each were used in the test. Readings were taken after incubating at 37° overnight.

TABLE II.—*Showing Flocculation of Virus by Non-filterable Rous Tumour Preparations.*

Suspension tested.		Testing material diluted	1 : 1.	1 : 2.	1 : 4.	1 : 8.	0.
Purified N-F virus	+	N-F serum	++	++	++	+	+
,, ,, ,,	+	F ,,	+	+	+	+	..
,, F ,,	+	N-F ,,	++	++	++	..	—
,, ,, ,,	+	F ,,	?	—	—	..	..
,, ,, ,,	+	N-F extract	+++	+++	++	..	..
,, ,, ,,	+	F ,,	±	—	—	..	..
Crude N-F extract	+	N-F serum	+++	+++	++	++	+

The degree of flocculation is shown by the number of + signs.

The pH of all tubes was tested at the end of the experiment and found to be the same.

Gye and Purdy (1930*b*) reported that tumour extracts and extracts of normal tissues will sometimes turn cloudy on incubating for prolonged periods, and ascribed this to the action of an oxidase, noting that HCN, which prevents the oxidative destruction of Rous virus, will also inhibit the formation of the cloudiness. They noted, however, that the development of the cloudiness and the amount of destruction of the virus did not always run parallel, and that it was sometimes necessary to add HCN in concentrations which they had previously found to cause destruction of the virus in order to prevent the formation of the cloudiness. In the experiments described above, the action of enzymes would be prevented by the formalin present. It may therefore be concluded that the opalescence noted in tumour extracts may sometimes be due to antigen-antibody combination in addition to the factors such as oxidases which operate in extracts of normal tissues.

Absorption of Inhibitor.

In the case of a tumour whose activity has been reduced but not abolished by inhibitor action, it is to be expected that most of the antibody will be absorbed on the

virus particles and discarded when the cell-free extract is prepared by centrifuging. There is no reason to suppose that " inhibitor " will be similarly lost. This point was investigated in one experiment. A 28-day-old tumour was found to have its activity reduced to 5×10^4 agent particles per gramme of tissue, presumably by the action of inhibitor (see Carr, 1943*b*, for data on the increasing activity of inhibitor with age of tumour). A 10 per cent. cell-free extract of the tumour was heated to 56° for $1\frac{1}{2}$ hours to destroy the activity of agent without harming " inhibitor " (Murphy and Sturm, 1932*a*), but was found to have no effect on a purified Rous agent suspension. This demonstrates that the inhibiting material responsible for the reduced activity of the tumour was all lost in the preparation of the cell-free extract, presumably by the formation of floccules as described above. The similarity of this experiment to an ordinary antibody absorption experiment is obvious. This suggests that the whole of the " inhibitor " can be removed by absorption on tumour agent material, and suggests that only antibody was the cause of the reduced activity of this tumour.

Origin of Inhibiting Material.

Antibody formation is due only to the host. Though it is possible that a tumour may produce antibody, the question does not arise in the present circumstances for, in the first place, cell grafts of these non-filterable tumours did not continue to produce inhibitor (see Carr, 1942), and, secondly, this would not account for the production of inhibitor only in old tumours (Carr, 1943*b*). The following experiment was designed to demonstrate that all the action of the inhibitor encountered in this work can be ascribed to the host antibodies alone.

The chemically-induced non-filterable sarcoma GRCH/15 originated by Peacock was known to produce an extract which had no inactivating action on the Rous agent *in vitro* when the tumour was grown in young fowls (one experiment proving this is shown in Table I). It was also known that this tumour would grow in many birds immune to the Rous sarcoma (Carr, 1943*a*). Grafts of this tumour were made into the following four birds :

Fowl 658 : A bird taken at random from the Institute flock, whose agent-induced Rous tumours produced by inoculation 8.iii.43 had regressed.

Fowl 665 : Similar to the previous bird, agent inoculation performed 10.iii.43.

Fowl O.625 : Recovered from inoculation of tumour material made on 30.v.41. A high content of antibody to Rous agent was known to be always present in the serum.

Fowl O.1273 : Rous tumours regressed after inoculation made 13.viii.41. Not used for any other experiments.

All four birds were successfully grafted with GRCH/15 tumour cells on 13.v.43. As it has previously been shown (Carr, 1943*c*) that all birds after recovery from Rous tumours have a permanent high serum antibody content, antibody will be present in the GRCH/15 tumours in amounts that will be comparable with the amount in Rous tumours growing in similar birds. If anything, the GRCH/15 tumour, being more compact and non-haemorrhagic, will contain a lesser amount than would a Rous No. 1 tumour. Tumour material was taken from fowl 658 when killed 16.vi.43, from O.625 at death (due to visceral gout) 23.vi.43, and was removed by operation from the remaining two birds the same day. All tumours were microscopically and macroscopically entirely GRCH/15 in type. A 10 per cent. extract of each tumour was made, and tested for anti-virus activity by mixing with an equal volume of Rous agent suspension and inoculating into groups of susceptible chicks. It was found that the extract of fowl 658 completely inactivated 1000 M.I.Ds. of Rous agent, and

extracts of the remaining three tumours completely inactivated 100 M.I.Ds. All four extracts were devoid of tumour-producing activity when tested alone.

This demonstrates that the " inhibitor " action depends upon the host, and not on the tumour, and it is clear that the amount of antibody present in a tumour can be sufficient to inactivate all the agent that can be extracted from the tumour cells.

DISCUSSION.

In the present investigations into non-filterable Rous No. 1 tumours it has been shown that all birds bearing such tumours possessed a high antibody content in their serum, and that this alone is sufficient to inactivate free Rous virus. Also that the " inhibitor " causes flocculation of virus and can be absorbed from an extract by union with the virus—properties characteristic of antibodies. The antibody causes loss of activity in two ways, both by formation of floccules in the extract, with loss of agent in the preparation of the cell-free extract, and by inactivation of the remaining virus. It is apparent that these two mechanisms must have been responsible for some part of the activity ascribed to " inhibitor " by other workers. Though in this investigation it was found that this antibody alone was sufficient to produce all the inactivation of virus necessary to produce inactive tumour extracts, this does not rule out the presence of an additional " inhibitor " in the non-filterable tumours investigated by others. Nevertheless, many of the properties of the " inhibitor " reported in the literature are consistent with the view that it was this immunity action that was investigated. Thus Claude (1939) concluded that " inhibitor " was a protein destroyed by enzyme action. An increase in the activity of certain Rous tumour extracts had previously been found after enzyme action by Baker and McIntosh (1927), and by Fränkel and Mislowitzer (1930). Murphy and Sturm (1932*a*) found that " inhibitor " was inactivated at about the same temperature as Rous antibody, and that it could be removed by successive extraction of tumour material (*cf.* separation of virus and antibody by dilution shown by Andrewes, 1932). The separation of " inhibitor " and virus by centrifuging is paralleled by the separation of antibody and virus shown by Amies and Carr (1939). There are, however, two observations not in agreement with this conclusion. The experiments of Murphy and Sturm (1932*b*) on the effect of " inhibitor" on mammalian tumours are discussed above. Fränkel (1938) reported that " inhibitor" was extracted by lipoid solvents. This finding is contrary to the conclusions of all others who have worked on this subject, and the data that he offers in support of this conclusion is by no means decisive ; a confirmation of this work has not yet appeared.

The primary non-filterable Rous tumours became non-filterable because their slow growth enabled the host to produce an effective antibody concentration ; there is no reason to assume that this " inhibitor " action was a cause of their slow growth, as it is admitted that " inhibitor " action and rate of growth are not necessarily related (Murphy and Sturm, 1932*b* ; Carr, 1943*b*). The recurring Rous tumours owe their non-filterability to the antibody produced by the latent agent (Carr, 1943*c*). The time at which the " inhibitor " action becomes apparent, and the time at which it reaches an effective level (Carr, 1943*b*), are in agreement with the time expected for antibody production.

From this work on the Rous No. 1 sarcoma it can be concluded that a similar " inhibition " would also be found in the case of other virus-induced tumours. This is, of course, well known to be true of other fowl tumours, and a similar conclusion that antibody was responsible for the " inhibitor " of the Shope papilloma was reached by Friedewald (1939, 1940). It is therefore pertinent to call into question the majority

of experiments which have been performed to demonstrate the non-existence of infective viruses in tumours. The production of antibody alone may cause a sharp fall in the virus content of extracts of old tumours similar to that found for the Rous No. 1 (Carr, 1943*b*), and experiments on old tumours are thus invalid. Nor can it be agreed that any method of processing will eliminate the " inhibitor " effect if loss of virus by flocculation is not taken into account (*cf.* Murphy and Sturm, 1940).

Throughout this work only tumours from hosts in reasonably good health were used. This is an important restriction, as it is conceivable that severe ill-health of the host will cause a reduction in the amount of circulating antibody sufficient to allow a tumour to return to the filterable state. Such conditions are especially liable to occur with the more slowly-growing tumours. Furthermore, the expression " non-filterable," as applied in this work to the Rous No. 1 sarcoma, indicated that an extract failed to cause a tumour within three weeks when injected into susceptible birds, but workers who use more slowly-growing avian tumours often keep their animals under observation for many months. It is not always clearly recognized that this double standard of assessing filterability exists ; yet the difference is important, as some preliminary experiments (unpublished) have shown that Rous No. 1 tumour extracts that are non-filterable by the usual standard may yet cause tumours in a proportion of inoculated fowls after a delay of many months.

SUMMARY.

Serum antibody to the Rous No. 1 virus was always associated with the presence of " inhibitor " action, and is considered to be identical with it. The antibody causes a reduction in the amount of virus obtained from tumour extracts by forming floccules of virus and antibody which are lost in the preparation of cell-free extracts, and by neutralization of virus remaining in suspension. The amount of antibody contained in a tumour growing in an immune bird is sufficient to inactivate all the virus that could be extracted from the tumour cells. No evidence was obtained that a non-filterable tumour retained this property on transplantation into a non-immune host.

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The Tumour Virus disseminated from Rous No. 1 Tumours

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VI.—The Tumour Virus disseminated from Rous No. 1 Tumours.

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In common with other neoplasms, the Rous No. 1 tumour often shows small areas or even single cells that appear dead and disintegrating or moribund. These probably result from the interaction of such factors as inadequate vascularization, pressure, and the formation of non-viable cells as a result of abnormal mitosis. As death of the cell does not necessarily result in destruction of the tumour virus, the virus of such cells is presumably released into the host's tissues. There is some difference of opinion as to the part that this virus plays in extending the growth of the tumour by infecting neighbouring cells and by producing metastases (review in Foulds, 1934), but it is generally agreed that the virus is present in most tissues of an animal bearing a filterable tumour (Bürger, 1914; Fujinami and Suzue, 1925; Fränkel, 1927; Costa, 1932; Mellanby, 1935, 1936, 1937, 1938 *a*, 1938 *b*) without, however, producing any tumours. The reason for this absence of carcinogenic action on the part of the disseminated virus has remained obscure. It was also noted that recurring Rous tumours are always found at the sites inoculated with tumour material, and never result from disseminated virus (Carr, 1942). Mellanby (1938 *b*) noted that the amount at first increased and later decreased with time, no active virus being found forty or more days after the initiation of tumour growth in the host. A similar decrease in the amount of extractable virus in tumours has been described (Carr, 1943) and shown to be due to antibodies in the tissue fluid inactivating the virus as it is extracted from the tumour cells. This raised the possibility that virus was similarly present in the normal tissues of hosts bearing lasting tumours, though not demonstrable for the same reason. The present investigation was undertaken to see if this was the case, and to discover the reason for the absence of neoplastic action of such virus.

Experimental

Intracellular virus can be detected in the presence of considerable amounts of antibody by grafting the affected cells into a susceptible host. The antibody diffuses away, and the tumour cells, together with the host cells infected by virus from the grafted cells (most of which die), will then yield a progressive Rous No. 1 tumour. In this manner the presence of virus, and the amount of extractable virus, were determined on the spleens, and sometimes the livers, of birds inoculated with Rous virus. It is sufficient to describe the results obtained using spleen, which is reported to contain the highest concentration of disseminated agent (Mellanby, 1938 *b*). This observation was confirmed, and also the frequency with which these tissues yield active extracts, but the resulting picture was quite different from that gathered from a perusal of Mellanby's papers. The data obtained are summarized in the table. All hosts were Brown Leghorns of the Institute flock, inoculated at the age of six weeks with cell-free virus. Each testing inoculum was made into a group of 2–3 susceptible young chicks.

It will be seen that while inoculations of spleen cells and extracts both produced umours up to 29 days, after 42 days no tumours resulted, and the virus is no longer demonstrable. This contrasts sharply with the host tumour, whose cells always produced tumours, though extracts of old tumours failed to do so. It is apparent that the amount of virus in the spleen corresponds only to about 20 minimal infective doses per gramme, while Rous tumours in similar hosts contain 10^5–10^7 minimal infective doses per gramme (Carr, 1943). The amount is, in fact, just sufficient to ensure that inoculation of a cell-free extract will produce a tumour, and Mellanby's descriptions of the tissues as "rich in cancer-producing agent" and "contain

large quantities of cancer-producing agent" do not apply to these results. The amount of disseminated virus in the whole bird corresponds to the virus content of less than one milligramme of tumour.

TABLE I.—THE AMOUNT OF ROUS NO. 1 TUMOUR VIRUS IN THE SPLEENS OF INFECTED FOWLS

Inoculum	Duration of Tumour Growth in Host (Days)								
	13	18	24	29	43	49	63	94 *	181 †
0·01 g. spleen cells . .	+	+	+	+	−	−	−	−	−
Extract of 0·05 g. spleen .	+	+	−	+	−	−	−	−	−
,, ,, 0·005 g. spleen .	−	+	−	−	−	−	−	−	−
,, ,, 0·0005 g. spleen .	−	−	−	−	−	−	−	−	−
Tumour cells . . .					+	+	+		+
Tumour extract . . .			+		−	−	−		−

* Virus carrier; no detectable tumour.
† Recurring tumour.
A + indicates that a tumour developed in one or more of the birds inoculated.

DISCUSSION

From the earliest days of research upon the filterable fowl tumours it was reported, and repeatedly confirmed, that the blood of the host contains free virus (Rous, Murphy, and Tytler, 1912; Bürger, 1914; Pentimalli, 1916, 1924, 1934; Jablons, 1918; Lewis and Andervont, 1926; Fränkel, 1927; Ragnotti, 1929; Kusaki, 1930; Doerr, Bleyer, and Schmidt, 1932; Iida, 1933); a part of the 20 minimal infective doses will therefore be due to such virus. As it is recognised that the leucocytes contain more virus than the rest of the blood (Lewis and Andervont, 1926; Ragnotti, 1929), it seems plausible to suggest that the fixed phagocytic cells of the reticulo-endothelial system will also contain ingested virus particles; other viruses of the sarcoma-endothelioma-leukæmia series are known to be taken up by these cells. This would contribute to the fraction of the tissue-contained virus not removed by perfusion in Mellanby's experiments. If this is the case, then it would appear that practically all of the virus in the tissues is accounted for. This receives support from the relative abundance of the virus in various organs as determined by Mellanby, for most virus was present in organs in which phagocytic cells are common. Without further evidence, it thus appears that the regular occurrence of tumour virus in the non-phagocytic normal cells of the host is not yet conclusively established.

The fate of the phagocytosed Rous virus is uncertain, but it is probable that it is digested and destroyed, and thus fails to form a tumour. In this susceptibility to destruction it differs from the leukæmia viruses, but whether this alone is the main cause of the difference in action of the two types remains a matter for further investigation.

Finally, it might be pointed out that phagocytic action will be a complicating factor in "virus-absorption" experiments carried out with tissue suspensions, for which due allowance has not always been made.

SUMMARY

The amount of virus disseminated into the host's tissues from a developing Rous No. 1 sarcoma is only about 20 minimal infective doses in the richest tissue—the spleen. It is suggested that the whole of this can be referred to the amount contained in the blood and phagocytic cells present in the tissues.

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Lack of Transmission of Avian Tumour Virus from Carrier Hens to their Offspring via the Egg

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VII.—Lack of Transmission of Avian Tumour Virus from Carrier Hens to their Offspring via the Egg.

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(MS. received May 27, 1944. Read July 3, 1944)

THE frequent reports of 20 per cent. or more losses due to neoplastic diseases in poultry flocks indicate that this is one of the greatest troubles of the industry. Though certain of the avian tumours have been favourite subjects for experimental cancer research, little attention has been directed to the problem of the ætiology and control of avian cancer itself. Consequently, despite the urgency of the problem, there is little information available which can be used as a basis for prescribing any preventive measures. It is not even known whether the majority of the spontaneous tumours are induced by a virus. Not many attempts have been made to transplant and study these tumours, considering the wealth of material available; and only a small proportion of the attempts have succeeded. Though most of these successfully transplanted sarcomas were found to be associated with a causative virus, it is impossible to state whether this is the usual condition of spontaneous tumours, or whether the few successful transplants were aberrant forms whose associated virus altered the growth characteristics and thus enabled a successful transplant to be made more easily.

For some years the pedigreed Brown Leghorn flock of this Institute has been made available to workers on avian tumours, and as a result much valuable data has accumulated on the inheritance of susceptibility to various tumours. It has thus been shown that susceptibility to the Rous No. 1 filterable sarcoma is inherited (Greenwood, 1940), and that the resistant birds frequently respond to infection by this virus by producing only a small and transient growth or none at all (Carr, 1943 *a*). Yet the virus will remain latent in these birds for years after clinical recovery from the infection (Carr, 1943 *b*) and retains its power of causing malignant growths (Carr, 1942). Virus infection of such resistant birds would thus escape notice in a commercial flock, and the present work was undertaken to see whether these virus "carriers" might be responsible for the spread of malignant viruses in such flocks.

It has frequently been shown that fowl tumour viruses are disseminated about the body of a host bearing a freshly induced filterable tumour (Bürger, 1914; Fujinami and Suzue, 1925; Fränkel, 1927; Costa, 1932; Mellanby, 1938; Carr, 1944), and that secondary growths are especially liable to form in the functional ovary (Rous, Murphy, and Tytler, 1912; Ikeda, 1930). It is therefore to be expected that in such fowls the virus would pass to the egg, and hence to the offspring of the tumour host. Such transmission to the egg has been described by Mitsuo (1928), Oshima and Tomozawa (1931), and Ikeda (1930), and that this will result in infection of the embryo and hatched chick was established by Mio (1929) and Ikeda (1930). But the fowl bearing a progressive tumour has only a brief period of reproductive life left, and this is not very efficient (Ikeda, 1930; Carr, 1942), so that this mode of transmission of tumour viruses is of little practical importance. It is otherwise with the tumour virus "carrier" fowls. These appear to be quite healthy, and their laying capacity does not seem to be affected. Though no disseminated virus could be found in their tissues forty days after inoculation with the virus (Carr, 1944) the ovary was not examined. There does not seem to be any report of other virus diseases being similarly transmitted through the hen's egg to the hatched chick, but this is probably due to the fact that most virus infections of the hen have a rapidly fatal result, and the initial illness is sufficient to check laying entirely. On the other hand, transmission of serum immunity to the egg has been established from the early days of immunological research (Klemperer, 1893), and Andrewes (1939) found that the immunity of birds "naturally immune" to the Rous No. 1 sarcoma would pass to their eggs, the immunity being entirely localised in the yolk. The "carrier" birds also have a high serum titre of virus neutralising antibody (Carr, 1943 *b*). The relationship between this "carrier" immunity and the "natural antibody" sometimes found in birds is

not known, but it would seem likely that the immunity of the carriers would be passed in a similar fashion to the eggs, as the egg-white contains little but albumin, while the yolk protein livetin is similar to, if not identical with, the serum globulin (pseudoglobulin) (Jukes and Kay, 1932), with which is also associated the neutralising antibodies to the Rous virus (Andrewes, 1932). Accordingly, the neutralising ability of the eggs of the carriers and of normal hens was first investigated.

Experimental

The test for the presence of virus-neutralising antibodies was carried out in the same way as is used for similar tests with serum, by mixing an equal volume of the yolk or white with a partially purified suspension of virus in saline. In all experiments a concentration of 1/10,000 HCN was maintained, to protect the virus from oxidative destruction. After incubation for 1 hour at 39° the suspensions were inoculated intramuscularly into groups of 2–3 young chicks; the size of the resulting tumours is proportional to the amount of un-neutralised virus in the inoculum. As the high serum antibody of the carrier fowls is constantly maintained, a test on a single egg should be sufficient to indicate whether an appreciable amount of antibody is constantly passed to the eggs, and thus with the chicks available it was possible to test a series of carrier and normal hens. The results were unambiguous. Albumin never produced any decrease in the tumour-producing power of a suspension, and was usually found to yield a product slightly more active than the control mixed with saline. This result can reasonably be attributed to the protective action of inert proteins on Rous virus suspensions (Gye and Purdy, 1930). The yolk of eggs from normal fowls similarly showed a slight protective action, but the yolk from carrier fowls invariably abolished the tumour-producing activity of the suspension of virus.

The complete details of one experiment are recorded below. As the remaining tests were carried out in essentially the same manner the results are merely summarised, in order to economise space.

Experiment I.—The tumour of fowl 727 was extracted, and the extract processed by the method of Amies and Carr (1939) to yield a concentrated stable suspension of partially purified virus in 0·9 per cent. saline which contained 1/10,000 hydrogen cyanide as an oxidase inhibitor. The suspension was diluted with cyanide-saline until 0·25 ml. was equivalent to 5×10^{-3} g. of tumour. Dilutions of this stock suspension were made in cyanide-saline for titration of the virus activity, and equal volumes of the stock suspension mixed with the yolk or albumin to be tested, or with serum of a young chick as control. After incubation for 1 hour at 39°, 0·5 ml. of each was inoculated into the breast or leg muscles of two groups of 3 young chicks in the manner shown in the table. Tumour size was recorded either when the bird had grown a tumour of about the maximal size possible, or after 27 days, when all birds were killed and examined. Tumour size was recorded by the number of + signs.

Table I.—Neutralising Activity of the Albumin and Yolk from the Eggs of Hens that are Carriers of the Rous No. 1 Tumour Virus

Inoculum	Site Inoculated	Fowl No.		
		742	743	744
Virus suspension + normal serum	Right breast	+ + +	+ + +	+ + + +
" " + egg albumin from O.625	Left breast	+ + +	+ + +	+ + + +
" " diluted 1/100	Right leg	+ +	+ +	+ + +
" " " 1/1000	Left leg	–	–	–
		745	746	747
" " + normal serum	Right breast	+ + + +	×	+ + + +
" " + egg yolk + albumin from O.619	Left breast	+	×	+ +
" " + egg yolk from O.1307	Right leg	–	–	–
" " + egg albumin from O.1307	Left leg	+ + + +	×	+ + + +

× Small tumours appeared and regressed.

The results of several such determinations of the neutralising power of the yolk and albumin of both carrier and normal hens are summarised below.

NORMAL FOWLS: ALBUMIN

Albumin from the eggs of 2 normal fowls did not reduce the tumour-producing power of suspensions containing 50 minimal infective doses (M.I.D.s) of virus.

NORMAL FOWLS: YOLK

Yolk from the eggs of 4 normal fowls did not reduce the tumour-producing activity of suspensions containing 100 M.I.D.s of virus.

CARRIER FOWLS: ALBUMIN

Albumin from the eggs of fowls O.138 and O.236 did not reduce the tumour-producing activity of suspensions containing 50 M.I.D.s of virus; albumin from the eggs of fowls O.625, O.1274, O.1307 did not reduce the tumour-producing power of suspensions containing 100 M.I.D.s of virus.

CARRIER FOWLS: YOLK

Yolk from an egg of fowl O.138 completely destroyed the tumour-producing activity of a suspension containing 50 M.I.D.s of virus; yolk from the eggs of fowls O.1240, O.1274, O.1300, O.1307 completely destroyed the tumour-producing activity of suspensions containing 100 M.I.D.s of virus.

CARRIER FOWLS: ALBUMIN + YOLK

A mixture of three parts of albumin to one part of yolk from an egg of fowl O.619 much reduced, but did not entirely abolish, the activity of a suspension containing 100 M.I.D.s of virus.

All carrier birds had been inoculated with Rous No. 1 sarcoma virus at the age of 6 weeks in 1941. All tests on eggs were carried out in the latter half of 1943.

The high concentration of antibody in the yolk will render it impossible to demonstrate any virus that may be present. But as neutralising antibodies eventually cause virus destruction (Andrewes, 1932) such virus, if present, will be rendered ineffective. The results obtained with albumin also suggest that, if any virus is present in the albumin of the carriers, it is only small in amount, otherwise the activity of the albumin + suspension mixture would have been greatly increased. Albumin from the eggs of carriers was accordingly tested, but failed to induce tumours when inoculated into susceptible chicks, as also did the chalazas of such eggs. One egg was tested from each carrier.

CARRIER FOWLS: ALBUMIN

0·5 ml. of albumin from the eggs of carrier fowls O.135, O.619, O.1268, O.1300, O.1307, 2410 failed to induce tumours in susceptible chicks.

CARRIER FOWLS: CHALAZA + ALBUMIN

One chalaza together with adhering albumin taken from the eggs of carrier fowls 840 and 1167 failed to produce tumours in susceptible fowls.

Fowls whose number is prefaced by the letter "O" were inoculated in 1941, the others in 1942. These tests of eggs were carried out in the latter half of 1943.

CARRIER FOWLS: EMBRYOS

This absence of transmission of the virus to the eggs is confirmed by results obtained in breeding experiments using carrier fowls. The hatchability of the eggs of such fowls is not abnormally low, and of 12 cases of "dead in shell" that were examined no neoplastic condition could be recognised. This is not decisive evidence of the absence of the virus, as it can often remain latent in its use without producing tumours, and fails to exert its neoplastic activity in adults without a concomitant injury (Rous, Murphy, and Tytler, 1912; Pentimalli, 1924; Doerr, Bleyer, and Schmidt, 1932). Accordingly two 10-day embryos derived from carrier fowls were minced and inoculated into 5 susceptible chicks. The resulting "embryomas"

grew and almost completely regressed at the same rate as those formed from embryos of normal fowls. Histological examination at the height of growth failed to show any Rous No. 1 sarcoma tissue, and the small growth left after regression consisted, as usual, mostly of cartilaginous material, and contained nothing resembling sarcoma tissue.

Carrier Fowls: Chicks

Some hundreds of chicks have been raised from carrier fowls in the course of breeding experiments. Though most of these chicks were, like their parents, resistant to the Rous virus, the resistance was never complete in all cases, and some were capable of growing persisting Rous tumours. The proportion of chicks thus reacting varies according to the age of the chick (Carr, 1943 *a*). Because of the excitable and pugnacious disposition of the young chick trivial injuries are frequent, and these could serve as sites for the neoplastic action of the virus to become manifest. However, not a single example of any neoplastic condition has been observed in uninoculated chicks up to the age of 6 weeks, nor is any serum antibody then found, indicating that they are not carriers (Carr, 1943 *a*). Thereafter the majority are used for experimental purposes, and, as stated above, a proportion is found to develop progressive tumours after artificial infection with the Rous No. 1 virus.

Carrier Fowls: Infection of Neighbours

From the beginning of work on the filterable fowl tumours it has been recognised that tumour-bearing fowls will not spread the disease to other fowls by contact (Rous, Murphy, and Tytler, 1912). As may, therefore, be expected, other fowls present in the breeding pen containing carrier fowls have never developed Rous No. 1 tumours, though contact has in some cases extended over a period of years.

Discussion

The results indicate quite clearly that there is no transmission of the infective virus *via* the egg in the case of the Rous No. 1 sarcoma virus. This parallels the failure to find the virus disseminated into the organs of such fowls (Carr, 1944). To what extent this can be applied to other viruses is uncertain, but the result suggests that such transmission may be unlikely as far as viruses similar to the Rous No. 1 are concerned. However, the possibility remains and the existence of hypothetical viruses with a special affinity for the ovarian tissues must be taken into account. But the present investigation does not suggest that transmission *via* the egg is an important cause of the high incidence of neoplasms in poultry.

Furthermore, no transmission of the virus from carriers to other birds has been found. Yet this result should only be transferred to other flocks with caution, for the conditions that obtain in the Institute flock may have influenced this result. It is curious that the flock having been raised free from parasites and infectious diseases, it was also to a very great extent found to be free from neoplastic diseases. It is thus possible that the careful husbandry employed has eliminated some factor that operates in the ordinary flock.

This investigation has established that the carrier hen will transmit virus-neutralising antibodies to the yolk of the eggs laid by her. This must represent a very considerable drain upon her antibody resources, yet a laying hen will maintain a high serum titre of antibody (Carr, 1943 *b*). The latent virus in these birds must thus be in a condition and situation to stimulate antibody production with great efficiency, which makes a remarkable contrast with its failure to induce neoplastic growth at the same time.

The transmission of the antibody of the carrier to the egg is paralleled by the observation of Andrewes (1939) that the "natural antibody" found in some old fowls is similarly transmitted. Andrewes noted that this antibody failed to influence the susceptibility of the chick derived from such eggs, and that it vanished in 4 weeks, and failed to return. Similarly, the carrier antibody must vanish from the chicks raised from the eggs of carrier hens by 6 weeks of age, for at that time the inoculation of a low infective dose of tumour virus will produce tumours (Carr, 1943 *a*). It is recognised that this serum antibody has little influence upon the growth of established tumours, as it fails to pass the cell wall, and cannot affect the virus. Yet at the age of 6 weeks a considerable resistance to tumour growth may be evident, and this

resistance is inherited and independent of humoral immunity reactions (Carr, 1943 *a*). It must thus be concluded that this transmitted antibody is of little importance in affecting the resistance of the birds to the action of neoplastic viruses.

SUMMARY

Hens that are carriers of the Rous No. 1 sarcoma virus lay eggs that contain a considerable amount of virus-neutralising antibody in the yolk. Virus cannot be detected in the egg, embryo, or chicks derived from such birds. Carriers did not infect other birds in the same pen with the sarcoma virus.

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Action of Notatin on the Rous No. 1 Sarcoma Virus

As notatin exerts its antibiotic activity by virtue of the hydrogen peroxide produced by its oxidation of glucose to gluconic acid[1], it seemed worth while to try the action of this material upon the Rous No. 1 sarcoma, the causative virus of which is readily destroyed by oxidation[2].

A specimen of notatin, active at 1 in 500,000,000 against *Staph. aureus* when last assayed, was generously provided by Dr. J. H. Birkinshaw. It was found that 0·2 mgm. of notatin, added with 2 mgm. glucose to 0·5 ml. of a suspension containing 1,000 minimal infective doses of Rous No. 1 sarcoma virus partially purified according to the method of Amies and Carr[3], resulted in almost complete loss of activity of the virus in 1½ hours, in two separate experiments. Notatin alone caused a slight reduction in the activity of similar suspensions, while glucose alone had no effect.

In contrast to the activity of notatin *in vitro*, it was found to have no action upon the virus *in vivo*. Amounts ranging from 2 mgm. to 5 mgm. were inoculated into one tumour of six fowls bearing three or four Rous No. 1 sarcomas in various sites, but it was found that all tumours continued to grow, and the size of the treated tumour relative to the others was not decreased. Doses of 8–10 mgm. were fatal to the nine-week-old Brown Leghorns employed. Similarly, pre-treatment of fowls with notatin failed to influence the tumour-producing action of sarcoma virus injected shortly afterwards. Two groups of three birds were injected with either 2 mgm. or 5 mgm. of notatin into the left breast, and 1½ hours later each breast was inoculated with about 200 infective doses of sarcoma virus, and the legs with about 10 infective doses. No difference in size was found between the sarcomas produced in the notatin-treated breast and those induced in the untreated side; and the small dose injected into the legs produced tumours in all birds, indicating that a systematic reduction in infectivity of all virus injected into notatin-treated birds had not occurred.

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Mechanism of the Feulgen Reaction

THE Schiff reaction for aldehydes using the addition compound of sulphurous acid with fuchsine was applied by Feulgen for his well-known reaction for the localization of desoxyribosenucleic acid in cells. The specificity of this localization in the reaction has recently been questioned[1], and led to the series of investigations to be described, from which it is concluded that the reaction is essentially one of adsorption, and that nucleic acid is not necessarily concerned in the reaction. Normal, malignant and embryonic tissues of mouse, fowl and normal rabbit tissues, fixed by various methods, were used in the staining tests.

(1) *Colour of the stain.* It can readily be shown that though neutral fuchsine stains filter paper or sections bright red, the typical mauve colour is produced if the dye is used in weakly acid solution, as in the usual Feulgen method. Though the colour is washed out of filter paper by sulphur dioxide water, it is not removed completely from other materials such as newsprint, poor quality cotton wool, or alumina. Furthermore, a section stained bright red by neutral dye will show the typical Feulgen colour if dipped into acid buffer solution. The colour does not therefore indicate anything about the nature of the material.

(2) *Hydrolysis.* In the Feulgen reaction the tissue is placed in $N/1$ hydrochloric acid for a few moments 'to hydrolyse the nucleic acid'. But acid is known to destroy much of the cytoplasm[2], though the nucleus remains intact for a longer time in the same circumstances. The method is, in fact, used to prepare free nuclei for analysis. An examination of finely minced tissues subjected to such treatment for varying intervals will leave no doubt that very extensive destruction of the cytoplasm occurs during hydrolysis, and will suggest that the localization of the stain in the nucleus in the Feulgen reaction may well be due to the fact that only a ghost of the cytoplasm is left after hydrolysis. Before hydrolysis both the cytoplasm and nucleus of a section stain mauve with acidified fuchsine, but after hydrolysis only the nucleus takes the stain; the appearance is then exactly the same as that of a normal Feulgen stain for such tissue.

(3) *Staining of chromosomes.* The Schiff reaction is carried out in solution. When a solid phase is present, as in the Feulgen reaction, complications due to adsorption may occur. If an adsorbing material is present which will disrupt the sulphurous acid–fuchsine compound by having a stronger attraction for the dye than the sulphurous acid, the mauve

colour will reappear. This can be shown in a test-tube reaction by adding alumina to Schiff reagent, when the dye (in the coloured form) is adsorbed on the solid, even in the presence of a large excess of sulphur dioxide. As it is well known that chromosomes will adsorb and concentrate dye from a weak solution, this effect cannot be disregarded in the Feulgen reaction. In fact, the varying distribution of the Feulgen stain in chromosomes with heterochromatic regions is equally well shown by certain simple stains such as crystal violet, where only differential adsorption need be considered.

The degree to which adsorption acts in the Feulgen reaction can be found by adding a large excess of sulphur dioxide to the Feulgen stain, and staining as usual. Thus in one experiment there was more than forty times the wet weight of the section of sulphur dioxide in excess ; even if the section were all nucleic acid, there was not enough to act with the sulphur dioxide present to liberate any colour. Nevertheless, a perfectly normal 'Feulgen reaction' was obtained. Similarly, if the section is pretreated with sulphur dioxide water before immersing in the stain, this sulphur dioxide will react with all the desoxyribose, and thus no further reaction can be expected when the section is placed in the decolorized fuchsine solution. In spite of the fact that no dye can thus be liberated by chemical reaction, here again a perfectly normal 'Feulgen reaction' results.

It seems clear, therefore, that neither the presence nor the location of the stain bears any relation to the presence or absence of either of the two nucleic acids, but can be merely a reflexion of the adsorbing power of the chromosomes. It should be noted that the adsorbing reactivity is only developed after hydrolysis. This different adsorbing power, with which is associated differences in the biological activities of the parts of the chromosomes, cannot therefore be proved to be due to differences in nucleic acid concentrations or different varieties of nucleic acid (ribose or desoxyribose types) by the use of the Feulgen reaction. For the demonstration of these adsorption differences in the chromosomes the use of acidified fuchsine on acid-digested material is more convenient than the complications of the Feulgen reaction using decolorized fuchsine.

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THE TUMOUR VIRUS DISSEMINATED FROM FILTERABLE TO NON-FILTERABLE TUMOURS.

by

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From The Institute of Animal Genetics, University of Edinburgh.

It was recently shown that under certain circumstances the virus of the Rous No.1 sarcoma can remain latent in the tissues of the host without exerting any neoplastic action (Carr, 1943b). This latent state recalls the experiments of Mellanby (1938), who demonstrated that when a Rous No.1 and a non-filterable tumour are growing in the same host, the virus of the filterable tumour can often be detected in cell-free extracts of the non-filterable tumour, though the presence of the virus is not indicated by any alterations in the growth-rate or histological and cytological characteristics of the infected tumour. This transfer of virus to a non-filterable tumour was also found by Gye and Schlesinger (unpublished), who found that the virus of the MH/2 endothelioma would pass to a chemically-induced fibrosarcoma in the same host, the identity of the virus being indicated by the characteristic MH/2 histology of the tumour resulting from inoculation of the fibrosarcoma extract. Similarly, a Fujinami tumour passed a virus to non-filterable tumours which could be identified as Fujinami by its ability to infect ducklings. It is uncertain,however, whether such virus is in the ineffective "latent" condition in virus-sensitive cells, or whether it is merely the virus known to be constantly

disseminated from filterable tumours into the blood and thus into the phagocytic cells of the host's organs, in which situations its neoplastic activity does not become manifest (Carr, 1944). If the virus content is due only to such disseminated virus, then only a small amount will be present; the actual quantity depends upon such factors as the volume of blood and number of phagocytic cells present in the tissue, but even in the richest known source, the spleen, it seldom exceeds 20 minimal infective doses (M.I.D.s) per gramme of tissue (Carr, 1944). On the other hand, if many of the tumour cells are involved, a much greater content would be anticipated. Assuming a volume of $(10\mu)^3$ per cell, then if only 0.1% of the cells were to contain a single associated tumour virus particle, about 10^6 particles per gramme would be present.

The experiments of Mellanby were not designed to indicate the actual concentration of tumour virus contained in the non-filterable tumours, but there was some suggestion that only a small amount was involved. In the present investigation, quantitative determinations of the contained virus were made, in the hope of deciding between the two possibilities.

METHODS

The experiments were carried out using Rous No.1 sarcoma as the filterable tumour, and two non-filterable chemically-induced avian sarcomas, the GRCH/15 originated by Peacock and generously made available by him and

a methylcholanthrene-induced sarcoma Mca 1 originated at this Institute, and which it is hoped to describe in a subsequent publication. Grafts of either of the two non-filterable tumours were made into the breast muscles of chicks of the Institute flock aged 6 - 9 weeks and some time later the birds were inoculated with virus of the more rapidly-growing Rous No.1 sarcoma in each leg. After a suitable time had elapsed, the bird was killed, and a portion of each type of tumour was minced with scissors, extracted for some hours with 10 volumes of M/100 Na_2HPO_4 solution containing cyanide as oxidase inhibitor, and the extract clarified by centrifuging. Ten-fold dilutions of each extract were prepared, and the virus content determined by intramuscular inoculation of 0.5ml into groups of 3 young chicks.

RESULTS

The results are summarised in Table I.

Table I. - Virus content of filterable and non-filterable tumours growing in the same host.

Expt. No.	Rous tumour age (days)	Virus content M.I.D.s/g	Non-filterable tumour	age (days)	Virus content M.I.D.s/g
1	11	>200,000	GRCH/15	33	0
2	20	> 20,000	"	43	20
3	26	> 20,000	"	49	0
4	27	> 20,000	"	63	20
5	7	> 200,000	McaI	39	0
6	13	> 200,000	"	46	0
7	17	> 200,000	"	128	0

It will be seen that the non-filterable tumour sometimes contained a small amount of the Rous No.1 virus, but that none was found in most experiments. A negative finding indicated that in 3 x 0.5ml of 10% tumour extract no virus was detected, i.e. there was less than 1 M.I.D. in 0.15g of tumour. It seems that the small amount sometimes found can reasonably be attributed to the vascular portions of the tumour tissue, rather than to the tumour cells themselves. In agreement with Mellanby's findings with other tumours, the contained virus did not affect the gross or microscopic appearance of the non-filterable tumour, which further suggests that it was not associated with the tumour cells.

It is possible to raise the objection that the tissue debris of the non-filterable tumour that is discarded after centrifuging had absorbed a quantity of Rous No.1 virus contained in the tumour. In order to control this possibility equal portions of filterable and non-filterable tumour taken from sites adjacent to the parts removed for extraction were minced together, extracted in the same way as the other tumours, and the activity compared with that of the extract of Rous No.1 tumour alone. No appreciable decrease was noted in two experiments with GRCH/15 tumours, two experiments with Mca I tumours or two similar experiments in which a GRCH/15 and an McaI tumour were taken from hosts not growing a Rous tumour. Loss of virus by such absorption can therefore be disregarded.

Discussion

It will be noted that the earlier growth of the non-filterable tumour

in the host did not appear to reduce the virus content of the extract of the Rous No.1 tumour later inoculated in the same host. It has been shown Carr, (1943a) that a developing Rous No.1 tumour will immunise the host against its contained virus in about 40 days. Consequently the above result suggests that neither the GRCH/15 nor the Mca I tumour contain an antigen which will immunise a fowl against the Rous No.1 virus. This suggests that as far as the fowl is concerned, there is nothing in common between the Rous No.1 virus and the two chemically-induced sarcomas employed. This is in contrast to the cross-neutralisation observed by Gottschalk (1943) with the filterable sarcoma 13 and non-filterable sarcoma 16. Other workers have shown that cross-neutralising sera can be obtained when the antibodies are produced in foreign species, but as similar relations can be shown between the virus and normal fowl tissues (Gye and Purdy, 1931), the significance of these findings is not easy to evaluate, and no attempt was made to carry out such investigations with the present material. This work does not indicate any attraction between the tumour virus and either the intact or damaged non-filterable tumour cell. Either such cells lack the specific receptors for the virus (suggesting that they are a different type of cell to that attacked by the Rous No.1 virus) or the receptors are present but inactivated. A considerable amount of "heavy protein" is associated with both GRCH/15 and McaI, and as Claude (1940) has shown that such material in many ways resembles the tumour viruses, the possibility that any receptors present are saturated with such "inactive

virus" cannot be ignored.

Summary.

Only small amounts of tumour virus are present in non-filterable GRCH/15 and McaI tumours growing in the same host as a Rous No.1 tumour and is attributed to the virus disseminated about the vascular system of the host. The cells of neither of the non-filterable tumours will absorb the tumour virus, nor do they contain an antigen producing antibodies to Rous No.1 sarcoma virus in the fowl.

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An Unexplained Discrepancy between the Actual and Expected Yield of Virus from Avial Tumours and its Implications.

by

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For many years now the filterable viruses have been considered as a possible cause of cancer, though such a theory has never found favour with the majority of pathologists. Definite proof of a causitive virus has, in turn, been found for many avian tumours (review in Foulds, 1934), rabbit fibromas (Shope, 1932), rabbit papillomas, (Shope, 1933), frog kidney tumours (Lucke, 1938), and the mammary tumours of mice (Bittner, 1937), and there is some evidence that certain other mouse tumours may depend on the presence of a milk-transmitted factor similar to that responsible for the mammary carcinomas first investigated. For many other types of neoplasms there is a greater or lesser amount of negative evidence for the existence of causative viruses, but until the true aetiology of these tumours is discovered, it is impossible to disregard entirely the possibility that a virus-like entity may play a part, and arguments for and against continue to be urged by both sides. Two excellent modern statements of the case for a virus aetiology of neoplasms are given by Rous (1943) and Oberling (1942). An essential point in this argument is to offer reasons why the virus may be difficult or impossible to demonstrate in spontaneous neoplasms. Among these may be mentioned Andrewes' (1939) conception of a non-infective

"toothless" virus; another is the well-known fact that even the classical virus-induced tumour, the Rous No.1 sarcoma, often produces tumours in which it is impossible to demonstrate the presence of the virus (e.g.Gye and Andrewes, 1926; Carr, 1942, 1944). An extreme example is that of the mouse mammary tumours, in which the presence of a causative, self-reproducing virus-like agent remained undetected during 3 decades of intensive research. Recently it was shown (Carr, 1944) that the Rous No.1 tumour virus can be detected only in young tumours, as the host rapidly produces antibodies sufficient to neutralise the virus in extracts of old tumours. This antibody does not affect the growth of the tumour, as it fails to penetrate the cell wall. As was pointed out, this is likely to be true of other hypothetical viruses, so that negative evidence of their existence is of little value if the experiment was carried out upon old tumours; unfortunately this has usually been the case. A further possible difficulty invoked as an explanation for the failure to demonstrate a virus in tumours is that the best-known tumour viruses, those of the avian sarcomas, are known to be readily destroyed by oxidation (Gye and Purdy, 1930); it may happen that other hypothetical viruses, having a slightly greater sensitivity to oxidative destruction, cannot be isolated unharmed by the means at our disposal. Also, the virus of the Shope papilloma usually cannot be extracted from the growths (papilloma or carcinoma) that it induces in domestic rabbits, though it can be detected by appropriate serological methods (Kidd et al.,1936).

It is the purpose of the present communication to raise another point bearing upon this difficulty of demonstrating tumour viruses; this is that the amount of virus experimentally found in the avian virus-induced tumours is entirely inadequate in amount to fit the facts as required for the conventional theories of virus induced tumours.

It is conventionally assumed that the Rous No.1 virus infects an injured cell of the monocyte or related type and converts it into a typical sarcoma cell. In the unrestrained mitosis that follows, the virus, which also multiplies, is distributed among all the descendants of the infected cell. This requires that there should be at least one virus particle per cell in the tumour. There have been a few reports of "inclusion bodies" in the tumour cells of certain avian tumours (Sanfelice, 1927, Turevich, 1939; Tenenbaum and Doljanski, 1941), but it is not known whether these represent aggregations of the infective virus. But these observations nevertheless suggest that there is some local concentration of the infective particles at these points of the cell, and that in these cells at any rate, there is a greater amount of virus than merely one particle.

A content of one virus particle per cell is therefore probably an underestimate. Yet by assuming this, and that there is one sarcoma cell to each $(10\mu)^3$, it follows that there must be $100^3 = 10^6$ virus particles per mm^3, or 10^9 per cm^3 (or per gramme of tissue).

Experimental determinations of the virus content of avian tumours have never reached anything like this figure. Unfortunately most workers

fail to employ quantitative methods in their experiments, but most of the later data available are summarised below. All results are the highest figure reported, recalculated as yield per g. of tissue.

Baker and McIntosh	1927	2×10^4 (Rous No.1)
Gye and Purdy	1931	Data for many tumours, number not specified. Maximal content 2×10^5 (Rous No.1)
Doerr, Bleyer and Schmidt	1932	4×10^4 (Rous No.1)
Amies	1937	8×10^5 per gramme (Rous No.1) 8×10^5 per gramme (Fujinami)
Elford and Andrewes	1935	2×10^4 (Rous No.1) 2×10^4 (Fujinami)
Fränkel and Mawson	1937	2×10^4 (Rous No.1)
Pollard	1938	10^5 (Rous No.1)
Amies and Carr	1939	4.8×10^5 per gramme (Rous No.1)
Shemin, Sproul and Jobling	1940	4×10^5 (Rous No.1)
Claude and Rothen	1942	3.75×10^7 (Rous No.1)
Carr	1943	Data for 61 tumours. Maximal value shown to be in young tumours growing in young chicks. Maximum virus concentration 5×10^6 virus particles per gramme (Rous No.1)
Carr	Unpublished	5×10^6 (Des.Legneris)

From this it is seen that the best recovery so far reported is that of 3.75×10^7 infective doses per gramme (giving 75% of takes), and if allowance is made for the cells other than tumour cells present in

bulk tissue, it is impossible to claim that more than 5×10^7 virus particles per gramme of tumour tissue have ever been proved to be present, and this figure may be too high. As the expected amount - at a conservative estimate of 1 infective particle per cell - is 10^9, the amount of virus actually found is at most only about 5% of the anticipated amount; the remaining 95% is unaccounted for.

If it must be confessed that in the case of the classical virus-induced tumours 95% of the virus cannot be demonstrated, then it is easy assumption that in other tumours, for similar unknown reasons, 100% may escape detection, and the transmission of the tumour apart from cells would thus be impossible to demonstrate by the usual techniques.

It is interesting to note that there does not seem to be comparable data for yield of infective virus per cell for any other viruses.

DISCUSSION

Several suggestions can be made to account for this enormous discrepancy between expectation and experiment, and it seems of value to examine what support is available for each of them, in order to obtain suitable orientations for new lines of attack upon the problem of the origin of cancer.

The simplest assumption would be that the missing virus is not there; in other words, that only 1 cell in every 20 contains a virus particle, and the remaining cells are malignant but devoid of the virus which caused the change. Were this the case, then it should be possible

to obtain a Rous tumour entirely devoid of virus, by transplanting such cells. There are, of course, many accounts of failure to demonstrate virus in extracts of avian tumours that are known to be caused by a virus; but in all cases so far examined, this has been due to an associated "inhibitor", which has been identified as anti-virus antibody. Because this action of antibody was not controlled, and as "natural antibodies" are often found in birds, the earlier reports of non-filterable extracts of filterable tumours cannot be accepted as evidence that virus was absent in the intact tumour cell. The fact that if these tumours were continued as cell grafts their own specific causative virus could later be detected suggests that the virus was merely "masked" and not absent (Carr, 1944). More support for this theory is perhaps provided by the observation of Fränkel(1927) that 50,000 cells never produce a tumour on inoculation, and 500,000 not constantly. While this type of experiment can be paralleled by similar experiments on tumours accepted as non-filterable, for there is an enormous mortality among transplanted cells, there is here the difference that a corresponding mortality of 50,000 virus particles must be assumed if each cell contains one virus particle. It is difficult to see how this could be so, as the conditions of grafting provide the best situation for infection by these viruses. Nor can any explanation be offered for the discrepancy between the loss of the virus from 50,000 cells needed for Fränkel's result, compared with the 1 in 20 needed by the data previously discussed.

Another possibility is that the error is due to the method of titrating the virus, and that more than one virus particle is needed to initiate a tumour; either a cell requires infecting by about 20 particles before a conversion to a malignant type is brought about, or only one in a 20 of the infected cells survives to produce the malignant growth. The latter explanation would seem to be disproved by the failure of several investigators to find large numbers of dead cells shortly after inoculation of the virus; while the results obtained when working at high dilutions of virus do not favour the former interpretation. On several occasions I have noted that very dilute suspensions of virus (less than 10 infective doses per inoculum) give a proportion of successful inoculations in about the proportions expected on the assumption that one particle is the infective unit. Furthermore, when several doses of such dilute suspensions are inoculated into the same animal the size of the tumours (which are proportional to the size of the infective dose of virus) becomes very irregular, though this irregularity is not found with a ten- or fifty-fold concentration of the same virus preparation. Such results are consistant with the expectation of the effects of random sampling on a small number of infective particles, but not as the variation of about 20 times this number.

Incomplete extraction of the virus from the cell remains a possibility. Against this may be set the experiments of Murphy and Sturm, (1932) where the yield of virus obtained by successive extraction of tumour tissue rapidly became negligible, and the fact that most workers have not noted an increased infectivity when the tumour cells are completely disrupted by desiccation or

the action of glycerol or multiple freezing and thawing. Nor will such an explanation account for the results of Fränkel discussed above.

There is also the possibility that much of the virus is in some way inactivated, and thus fails to form a tumour when injected into a susceptible animal. Considerable support for such a suggestion is provided by the observation that the product obtained by concentrating the active fraction of a tumour extract, though containing very much more material than is required for 10^7 virus particles, is nevertheless almost completely agglutinated by fowl virus-neutralizing antibody and flocculates at the same pH, and is therefore of a nature closely resembling the active virus. Yet against this is the difficulty of explaining how 95% of the virus can be inactivated almost immediately, and the remaining 5% is almost entirely unaffected for a comparatively long period. Oxidative destruction can thus hardly be responsible, the more so as Amies and Carr (1939) injected anti-oxidants into the host, thus ensuring that their action was present from the beginning of the experiment, and yet no great increase in the amount of virus recovered was noted. The least dry weight of virus preparation to induce tumours (75% induction) is 4×10^{-13} g by Claude (1942). The weight of the virus particle, assuming a diameter of 70μ (Elford and Andrewes, 1936) and a density of 1.3, is 2.3×10^{-16}g. This gives a ratio of 1 virus particle to 574 particles of inert matter, which is nevertheless immunologically and chemically indistinguishable from the virus, lacking only the property of infectivity. As one virus particle is derived from 20 cells, this gives a yield of 28.7 inert particles per cell. It does not seem

difficult to imagine that all this could be virus protein.

Another type of inactivation which may be considered is that due to free receptor groups such as often occurs in experiments with bacteriophage (Burnett, 1934; Levine and Frisch, 1934). It is known that the bacteriophage is attracted to specific receptor centres of the susceptible bacteria. These are of the nature of polysaccharides, and can become separated from the bacterial cell, to exist free in the suspending fluid. Phage particles can then become attached to these free receptors and so fail to infect the specific bacterium present. This would provide a satisfactory explanation for the missing virus of the avian tumours. While Fränkel claims that the lipoid fraction of tumour tissue contains an inactivating fraction other workers have failed to find such an effect (Sproul and Stevens, 1937; Pollard and Amies, 1938). Such hypothetical receptors, of course, need not be lipoil in nature.

Finally it may be possible that the virus is only infective during one small part of its life-cycle, or the mitotic cycle of the host cell. In the remaining period, it might be able to cause malignancy of the host cell, but be incapable of infecting another cell if removed from its host. It would then be in the form of a "toothless virus" in the sense of Andrewes (1939). There is immunological evidence that such types of non-infective virus is present in some non-filterable tumours (Andrewes, 1936; Foulds 1937; Gottschalk, 1943), and the existence of the two types of tumour cell in the Rous No.1 and related tumours (spindle cell and round cell) may reflect a similar variation in the virus.

A final possibility is that the virus used in extracts and suspensions may differ from the virus in the cell. The ability of proteins to aggregate to form stable multiples of an elementary molecule is well known, and the possibility cannot be excluded that cell-free virus is a complex of a smaller unit occurring in the cell; the participation of the host protein in such complexes cannot be disregarded, and this may explain the curious serological relationship found between these viruses and the fowl tissues (Gye and Purdy, 1931).

Many of these possible reasons for the absence of the virus are not mutually exclusive, and it is possible that the missing 95% of the virus may be accounted for by several of the possibilities outlined above.

SUMMARY

It is pointed out that the yield of virus from filterable fowl sarcomas at best corresponds to only one infective unit from 20 cells. With this is associated material physically, chemically and serologically indistinguishable from the virus, but approximately 574 times greater in amount. Possible implications of this are discussed.

All expenses in connection with this work were borne by the British Empire Cancer Campaign.

COMPARISONS BETWEEN THE MILK FACTOR AND FOWL SARCOMA VIRUSES

by

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Work upon these two types of carcinogenic viruses has usually proceeded independently, and by differing techniques. It is the purpose of this account to stress certain similarities in their mode of action. While genetic influences upon the action of the milk factor are readily shown by the use of pure line hosts, similar material is not available for the avian sarcoma viruses. But workers in this field have the advantage that the susceptibility of any animal can be immediately ascertained by injecting tumour virus; this is not possible in mice, as tumours arise only a long time after treatment with the virus.

There is a very high incidence of spontaneous tumours in ordinary flocks of fowls - veterinary post-mortem reports give up to 30% - and it is considered that most of these are caused by viruses. It is, therefore, not surprising that such birds are found to be 98% - 100% susceptible to the Rous No.1 sarcoma virus. As fowls can act as carriers of tumour virus, such birds are like the high cancer line mice, i.e. susceptible + latent virus. The Brown Leghorn flock raised by Dr. Greenwood has an abnormally low tumour incidence - less than 0.1%. These are equally sensitive to Rous 1 virus, and thus appear to resemble

fostered cancer mice in being susceptible, but devoid of carcinogenic virus. The susceptibility to Rous No.1 virus was found to be genetically determined, and Dr. Greenwood was able to produce a line of fowls so resistant to this virus that only about 10% produce progressive tumours upon inoculation. These fowls would thus correspond to low cancer strain mice. As in mice the virus can remain latent in such non-susceptible hosts. Maternal transmission of virus from such carriers does not occur in fowls, as it does in mice.

Carcinogenic chemicals will stimulate delayed tumour formation in virus-carrying mice and cause immediate tumour growth in virus-carrying fowls. Low doses of radiation also stimulate tumour production in carrier mice, while Peacock noted that when a Rous tumour was surrounded by radium needles, the tumour disappeared, but in the surrounding tissues, which received a lower dose of radiation, Rous tumours appeared immediately.

Both viruses are disseminated about the tissues of the host. In the fowls, this has been found to be due to the spread of the virus through the vascular system, and is small in amount; comparable studies for mice are not available.

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Heritable susceptibility of poultry to cancer virus

BY J. G. CARR, *Institute of Animal Genetics, Edinburgh*

There is a very high incidence of cancer in the domestic fowl of all breeds in every part of the world. It is usually considered that the majority of these infections are due to the action of carcinogenic viruses, of which a number are known.

Using the Rous No. 1 sarcoma as a test virus, and a 6–8-weeks-old chick as host, it was possible to show that the main factor in determining the extent of tumour growth was the genetic constitution of the host, and other factors were not involved to any great extent. Results obtained with the Brown Leghorn flock of Dr Greenwood are presented. From the data thus obtained, it was found possible to produce by selection a line of fowls exhibiting a very high resistance to the tumour virus. This resistance was not due to any transfer of inhibiting material through the egg, as indicated by breeding and other experiments. It was found that this line, though bred for resistance only to the Rous No. 1 virus, was also resistant to other carcinogenic viruses, and to a certain extent to chemically induced tumours.

The mode of transmission of the virus among fowls remains unknown. Effective transmission by the egg does not occur, and contact of normal birds with virus-infected fowls does not seem to cause infection of the normal fowls under conditions so far tried.